Up-regulation of proline-rich tyrosine kinase 2 in non-small cell lung cancer

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KEYWORDS
PYK2; Expression and activation; NSCLC; Metastasis; ERK1/2

Summary Proline-rich tyrosine kinase 2 (PYK2) is a non-receptor tyrosine kinase, plays different roles in intracellular signaling pathways, that regulates a number of biological processes, such as cell proliferation, differentiation, adhesion and migration, which have been shown to correlate with tumor development and aggression. However, the involvement of PYK2 in human non-small cell lung cancer (NSCLC) has not yet been determined.

In the present study, 90 patients with NSCLC (represented by adenocarcinoma and squamous cell carcinoma) were included retrospectively. NSCLC tissues were detected for the expression of PYK2 by immunohistochemistry. Correlation between the expression of PYK2 with the clinicopathological characteristics was analyzed. There were 64% (58 out of 90) of NSCLC patients with higher level of PYK2. Higher expression of PYK2 was significantly correlated with lymph node metastasis (node positive versus node negative, \( p = 0.007 \)). Patients with higher expression of PYK2 had advanced stage of NSCLCs (I + II versus III + IV, \( p = 0.012 \)). Protein level of PYK2 was also examined in 30 of these tumorous samples and matched non-tumorous counterparts by western blotting. PYK2 was apparently up-regulated in NSCLC tissues (tumor versus non-tumor, \( p = 0.000 \)). In the cell studies, extensive expression and activation of PYK2 were both found in higher metastatic BE1 cells. The activity of ERK1/2 in BE1 cells appeared extremely high as well. In conclusion, our results demonstrated that PYK2 is up-regulated in NSCLCs, and the higher expression and activation of PYK2 may play a role in modulating the activity of ERK1/2, and lead to the progression of NSCLC.

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1. Introduction

Lung cancer is the leading cause of death among the malignant tumors worldwide, and the incidence of lung cancer is increasing. Non-small cell lung cancer (NSCLC) is the primary histological classification of lung cancer, and the prognosis of patients with NSCLC principally correlates with tumor metastasis, which is regulated by associated gene

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expression. More information should be gathered on the research of prometastatic genes.

Proline-rich tyrosine kinase 2 (PYK2) is a member of FAK family, functions as an effector of multiple cytokines involved in the regulation of fundamental cellular activities, such as cell proliferation [1], differentiation [2], and motility [3]. PYK2 is up-regulated in different primary human tumors, including brain [4] and liver [5] tumors. Hepatocellular carcinoma patients with higher level of PYK2 had larger tumor size and advanced Edmonson grading [5], and PYK2 overexpression was found in invasive tumor cells and lung metastatic nodules by animal studies. The overexpression of PYK2 might play an important role in the progression of malignant tumors.

PYK2 is a non-receptor tyrosine kinase, and also an integrator for the signaling events that regulate tumorous processes of anti-apoptosis [6], adhesion [7] and especially migration [8]. PYK2 mediated biological functions are performed mainly by phosphorylation. PYK2 is rapidly tyrosine phosphorylated in response to various extracellular signals acting via different post-receptor pathways [9]. The tyrosine 402 (Tyr402) of PYK2 serves as the primary autophosphorylation site that is essential for PYK2 activation and function [10,11]. When activated by a number of cytokines, PYK2 leads to the activation of various downstream signaling molecules, such as ERK/MAPK [12–14], which results in the differential regulation of cell characteristics in different cell lines. The activity of PYK2 in Tyr402 may play an important role in up-regulating the migratory ability of tumor cells, which is supported by the high activity of Tyr402 found in the progression of breast tumor cells to have invasive and metastatic phenotype [15].

However, despite the increasing emphasis on PYK2 in human tumors, whether it positively participates in primary human non-small cell lung cancer has not yet been determined. The aim of this study is to investigate the expression and clinical significance of PYK2 in 90 surgically resected NSCLCs with different clinicopathological features, and the association of PYK2 expression and activation with ERK1/2 activity and metastatic potential of NSCLC cell lines.

2. Materials and methods

2.1. Tissue samples and patients

A total of 90 cases of non-small cell lung tumors were retrospective database from the Pathology Department of China Medical University. All of the enrolled patients underwent curative surgical resection without having chemotherapy or radiation therapy. Formalin-fixed paraffin-embedded sections of tumor obtained from surgical samples were stained routinely with hematoxylin and eosin (H&E), and reviewed by two senior pathologists in order to determine the histological type and stage, according to the WHO classification of lung and pleural tumors (2004) and the TNM staging system (1997). 30 cases (included in the 90 cases) of tumor and paired non-tumorous portion (distant from the primary tumor) of the same case were quickly frozen in deep freeze refrigerator until protein extraction. Lymph node status was determined by routine pathological examination of dissected pulmonary hilar and mediastinal and intrapulmonary lymph nodes. Clinicopathological information of the patients about tumor size, histological type, differentiation, stage and lymph node metastasis was obtained from patient records, and summarized in Table 1.

2.2. Immunohistochemistry

90 paraffin sections of tumor were deparaffinized and rehydrated routinely. The slides were then heated in an autoclave sterilizer for 2 min in 0.1 mol/L Tris–HCl buffer at pH 10. The sections were incubated overnight with primary rabbit polyclonal antibody detecting PYK2 (1:100 dilution, sc-9019) (Santa Cruz Biotechnology), following 3% H2O2 and

<table>
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<th>Table 1</th>
<th>Clinicopathological characteristics of 90 NSCLC tumors and PYK2 expression by immunohistochemistry</th>
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<tr>
<td>Clinicopathological characteristics</td>
<td>Cases (n = 90)</td>
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<tr>
<td>Tumor size and invasiveness</td>
<td></td>
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<tr>
<td>T1 + T2</td>
<td>47</td>
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<tr>
<td>T3 + T4</td>
<td>43</td>
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<td>Histological type</td>
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<td>Sq</td>
<td>48</td>
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<td>Ad</td>
<td>42</td>
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<tr>
<td>Differentiation</td>
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<td>Well</td>
<td>37</td>
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<td>Poor—moderate</td>
<td>53</td>
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<tr>
<td>Stage</td>
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<td>I + II</td>
<td>35</td>
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<td>Lymph node status</td>
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<td>+</td>
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<td>−</td>
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PYK2 expression: *significant difference between early (I + II) and advanced (III + IV) stage of NSCLCs; **significant difference between tumors with positive (+) and negative (−) nodes.
5% rabbit serum treatment at 37 °C for 1 h. After which they were incubated with secondary antibody and SP complex for 30min (SP kit-9710), lastly they were visualized with DAB (DAB kit-0031) (both from Maixin Biotechnology). Hepato-cellular carcinoma tissues were used as positive controls for PYK2, and negative controls were prepared by non-immune rabbit IgG at the same dilution as for the primary antibody. All the immunoreactions were separately evaluated by two senior pathologists. Brown particles appearing in cytoplasm was as regarded as positive cells. The intensity of PYK2 immunostaining (1 = weak, 2 = moderate, and 3 = intense) and the percentage of positive tumor cells (0% = negative, 1—50% = 1, 51—75% = 2, ≥76% = 3) were assessed in at least 5 high power fields (×400 magnification). The scores of each tumorous sample were multiplied to give a final score of 0, 1, 2, 3, 4, 6 or 9, and the tumors were finally determined as negative: score 0; lower expression: score ≤4; or higher expression: score ≥6.

2.3. Western blotting

Frozen tissues (including tumor and non-tumorous portion) or cells were washed twice with ice-cold phosphate-buffered saline (PBS), homogenized on ice in 10 volumes (w/v) of lysis buffer containing 20 mM Tris–HCl, 1 mM EDTA, 50 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 1% Triton-X100, 1 mM PMSF and phosphatase inhibitor using a homogenizer (Heidolph, DLA × 900). The homogenate was centrifuged at 15000 rpm for 30 min at 4 °C. The supernatant was collected and stored at −70 °C. Protein content was determined by the BCA assay (BCA protein assay kit-23227, Pierce Biotechnology). From each sample preparation, 80 μg of total protein was separated by 8% SDS-PAGE and then transferred to PVDF blotting membranes. The total protein extracts were analyzed by immunoblotting with indicated antibodies following SDS-PAGE analysis. Immunoblots were performed using rabbit polyclonal primary antibodies specific for PYK2, p-Tyr402 and β-actin (a housekeeping protein used as a loading control to assure equal amounts of protein in all lanes), and mouse monoclonal antibody for p-ERK1/2. After blocking nonspecific binding with 5% BSA in TBS (pH 7.5) containing 0.05% Tween-20 (TBST), primary antibodies were incubated on the membranes for PYK2 (1:300, sc-9019), p-Tyr402 (1:200, sc-9023), p-ERK1/2 (1:200, sc-7383) and β-actin (1:200, sc-1616-R) (all from Santa Cruz Biotechnology) overnight at 4 °C in TBST. Following three times washes in TBST, the membranes were incubated for 2 h at 37 °C with secondary goat anti-rabbit IgG antibodies (1:2000, ZDR-5306) and goat anti-mouse IgG antibody (1:2000, ZDR-5307) labeled with horseradish peroxidase (all from Zhongshan Biotechnology). Immunoreactive bands were identified using the DAB system (DAB kit-0031, Maixin Biotechnology), as directed by the manufacturer. Specific bands for PYK2, p-Tyr402, p-ERK1/2 and β-actin were identified by prestained protein molecular weight marker (SM0441, MBI Fermentas). The EC3 Imaging System (UVP Inc.) was used to catch up the specific bands, and the optical density of each band was measured using an Image J software. The ratio between the optical density of interest proteins and β-actin of the same sample was calculated as relative content and expressed graphically.

2.4. Cell lines and culture

Lung PG (pulmonary giant cell) giant cells including the higher metastatic BE1 cells and the lower metastatic LH7 cells [16] were kind gifts from Prof. Jie Zheng (Department of Pathology, Peking University, China). Human bronchial epithelial cells (HBE) and lung adenocarcinoma A549 cells were conserved in our department. BE1, LH7 and HBE cells were cultured in RPMI1640 and A549 was in DMEM (both from Gibco, Invitrogen Corporation) supplemented with 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin, glutamine, and NaHCO3, in incubator with 5% CO2. Cells were cultured to subconfluence until protein extraction. The experiments for each cell line were repeated at least three times.

Statistical analysis. SPSS for windows 13.0 statistical analysis soft was applied to complete data processing. χ2-test was applied to analyze the correlation between the expression of PYK2 and clinicopathological characteristics for the results of immunohistochemistry, paired-samples t-test was used to analyze the significant difference of PYK2 expression between tumor and normal, and independent-samples t-test was used to evaluate the difference of optical density in the neoplastic tissues with different node status as well as the lung cell lines with different metastatic potentials. Results were considered statistically significant at p < 0.05.

3. Results

3.1. PYK2 expression and localization in NSCLCs by immunohistochemistry

PYK2 immunoreactivity was detected in both normal and tumorous lung cells. In normal lung tissues, PYK2 expression was observed in ciliated epithelial cells, serous cells of the submucosal glands and alveolar cells, as shown in Fig. 1A–C.

PYK2 immunostaining was observed in every neoplastic tissue. We considered that 58 of the NSCLCs (64%) were higher expression (scores of 6 or 9); 32 cases (36%) were lower expression (scores of 0, 1, 2, 3 or 4), as described above in Materials and methods. PYK2 was also detected in the endothelial cells of vessels within the neoplastic stroma (Fig. 1D).

In addition, as shown in Table 1, no significant difference of PYK2 expression was found between the two most represented histological types of lung cancer (Ad versus Sq, p = 0.083). Examples of PYK2 immunostaining in adenocarcinoma and squamous cell carcinoma are shown in Fig. 1E–H. The negative controls were prepared by non-immune rabbit IgG at the same dilution as for the primary antibody, and results for normal and tumor samples were shown in Fig. 1I and J.

3.2. Comparative analysis and clinicopathological correlation

As shown in Table 1, no statistical difference was found between the higher PYK2 expression and the characteristics of tumor size (T1 + T2 versus T3 + T4, p = 0.102) as well as differentiation (high versus low, p = 0.605). However,
patients with higher PYK2 expression had advanced stage of NSCLC (I + II versus III + IV, \( p = 0.012 \)). In addition to promotion of cell proliferation, PYK2 also increased the tumor cells invasiveness and migration [20]. Therefore, the association between PYK2 expression revealed by immunohistochemistry and the presence of lymph node metastasis at the time of resection was analyzed statistically. Comparison of PYK2 expression was made between the NSCLCs with different lymph node status. PYK2 immunoreactivity was stronger in the NSCLCs with lymph node metastasis compared with the node negative cases, with moderate—strong immunostaining. Immunohistochemistry showed a statistically significant correlation between higher protein expression and a positive node (\( p = 0.007 \)).

### 3.3. PYK2 expression in NSCLCs by Western blotting

Western blotting was used to evaluate PYK2 expression in 30 NSCLCs and paired non-tumorous lung tissues distant from the primary tumor of the same case. The increased PYK2 expression was found in 27 NSCLC samples in comparison with the non-tumorous counterparts. The western blotting of eight samples is shown in Fig. 2A, and the optical density of the tumorous (T) and non-tumorous (N) tissues of the same patient was measured and expressed graphically (Fig. 2B). Previous researches have demonstrated that PYK2 overexpression enhanced cell mobility [8], thus comparison of PYK2 expression was made between NSCLCs with and

![Fig. 1](image1.png)

**Fig. 1** PYK2 expression by immunohistochemistry. (A) PYK2 immunostaining in the ciliated epithelial cells of bronchus. (B) PYK2 expression in the alveolar cells. (C) PYK2 immunoreactivity in the cytoplasm of serous cells. (D) PYK2 expression in the endothelial cells of neoplastic stroma. PYK2 immunostaining in lung squamous cell carcinoma with (E) and without (F) positive nodes. PYK2 expression in lung adenocarcinoma with (G) and without (H) node metastasis. It was shown that PYK2 expression was correlated with lymph node status, but not with histological type. Negative controls were prepared by non-immune rabbit IgG at the same dilution as for the primary antibody in normal (I) and tumor sample (J), respectively. Original magnification: all \( \times 400 \).

![Fig. 2](image2.png)

**Fig. 2** (A) Expression of PYK2 by western blotting in matched tumorous (T) and non-tumorous (N) tissues from 8 of 30 NSCLC patients, and 4 of which were accompanied with lymph node metastasis (the lower panel). Band intensities indicate significant PYK2 up-regulation in tumorous in comparison with the non-tumorous tissue of the same patient. Furthermore, patients with positive nodes expressed higher level of PYK2 in tumor. \( \beta \)-Actin was used as a loading control to assure equal amounts of protein in all lanes. (B) The ratio between the optical density of PYK2 and \( \beta \)-actin of the same patient was calculated and expressed graphically. The significant difference of PYK2 expression between tumorous (T) and non-tumorous (N) tissues as well as neoplastic samples with positive and negative nodes were analyzed statistically. PYK2 immunoreactivity is greater in neoplastic tissues (\( p = 0.000 \)) and cases with positive nodes (\( p = 0.018 \)).
Up-regulation of proline-rich tyrosine kinase 2 in non-small cell lung cancer

Role in promoting tumor cells more aggressive in NSCLC.

PYK2 is a non-receptor tyrosine kinase, mediates various biological processes, such as cell proliferation [18] and migration [19], all of which are critical to tumorigenesis, invasion and metastasis, and suggest a significant role of PYK2 in the development and progression of cancer [20]. PYK2 expression is up-regulated in a variety of human tumors including liver and brain tumors. Aiming at interfering PYK2 expression or blocking PYK2 activation may be helpful in the progression of effective anticancer therapies. Recent researches have been shown that inhibited PYK2 activity was correlated with the inactivation of ERK1/2, and reduced the adhesive ability of prostate cancer cells [7].

This study evaluated PYK2 expression in NSCLCs, with regard to the tumor size, histological type, differentiation, stage and lymph node status of NSCLCs, to determine the clinical significance of PYK2 for the advanced NSCLCs. We examined 90 tumors by means of immunohistochemistry, 30 of which were also analyzed by western blotting, and found a statistical evidence of PYK2 up-regulation in NSCLCs. Weak—moderate PYK2 immunostaining was observed in ciliated epithelial cells in bronchus, the serous cells of the submucosal glands, as well as normal alveoli. While PYK2 immunoreactivity was detected extensively in every neoplastic tissue, supporting the potential role in tumorigenesis, promoting proliferation and survival of tumor cells [5]. We also found that PYK2 overexpression was common in NSCLCs, regardless of the histological type and differentiation. However, patients with higher PYK2 expression had a significant metastatic phenotype.

The immunohistochemical observations may be further supported by our semi-quantitative western blotting evaluations of PYK2 expression in 30 tumors and paired non-tumorous counterparts. PYK2 expression were significantly higher in the neoplastic than the non-neoplastic tissues, which was consistent with previous studies of PYK2 overexpression in human malignant tumors [4]. Moreover, tumors samples in which PYK2 higher expression was determined by western blotting came from patients with lymph node metastasis at the time of resection. Taken together, these findings suggest that PYK2 up-regulation stimulated cell proliferation, and migration as well, possibly by attenuating the adhesive ability of tumor cells in NSCLCs [6].

Despite that further research is required, these results suggest a crucial role of PYK2 in affecting tumorigenesis and aggression of NSCLC.
Studies have been reported that PYK2 activity especially in Tyro42 in human malignant tumors has been correlated with outgrowth and invasiveness of breast cancer cells [9], and active ERK1/2 was included in LPA-induced PC12 cell migration mediated by PYK2 [3]. So we also examined the expression and p-Tyr42 of PYK2 and the p-ERK1/2 in the human bronchial epithelial cells (HBE) and different NSCLC cell lines. HBE cells as normal control, lower metastatic A549 cells as we confirmed previously, as well as lower metastatic LH7 cells and higher metastatic BE1 cells were included in this study. We found that the expression of PYK2 was quite weak in HBE cells, while the higher metastatic BE1 cells expressed the maximal level of PYK2, compared with the lower metastatic LH7 and A549 cells. So it is acceptable that PYK2 overexpression stimulated cell proliferation and more aggressive. Furthermore, PYK2 up-regulation was accompanied by the increasing PYK2 activity as evaluated by the level of p-Tyr42 [21], and ERK1/2 also proved to be more activated in higher metastatic BE1 cells. However, HBE cells expressed a low level of PYK2 with a scarcely detectable p-Tyr42 and a low p-ERK1/2. It was further indicated that PYK2 expression and activation might play a role in promoting a more malignant phenotype by regulating the activity of ERK1/2 in NSCLC.

Previous studies have been shown that PYK2 was essential for the pulmonary vascular endothelial cell spreading and migration [22], besides PYK2 kinase activity enhanced migration of endothelial cells [23], which resulted in vasculogenesis and angiogenesis. We also found PYK2 immunostaining in the endothelial cells of neoplastic stroma in several cases of NSCLCs, which showed lymph node positive. This pattern of PYK2 expression may indicate the invasive and metastatic malignant phenotype of NSCLCs.

Therefore, the PYK2 mediated signaling pathway may become a promising target for tumor inhibition. It has recently been shown that inhibiting PYK2 activity by the expression of kinase inactive PYK2 mutant attenuated the adhesive ability of prostate cancer cells [7]. The similar report has been found in glioma cells, in which the suppression of PYK2 activity within the FERM domain reduced the adhesive ability of prostate cancer cells [7]. The similar report has been found in glioma cells, in which the suppression of PYK2 activity within the FERM domain reduced the capacity of PYK2 to stimulate glioma cell migration [24]. As far as lung cancer is concerned, PYK2 appears to be the key interactive effector of Src in mediating the survival signals in lung adenocarcinoma cells upon detachment, which may contribute to the metastasis of malignant lung tumors [6]. Moreover, inhibition of PYK2 expression by siRNA, attenuated anchorage-independent survival and proliferation of SCLC cells [25].

In conclusion, our study demonstrates the facts that up-regulation of PYK2 is common in NSCLC tissues, increased expression and activity of PYK2 in lung cell lines are correlated with higher metastatic potential, possibly by the activation of ERK1/2. The detection of higher PYK2 expression by western blotting in 27 of the 30 NSCLCs examined suggests that PYK2 may play a role in promoting malignant transformation, which is crucial to the tumorigenesis of NSCLC. The suppression of PYK2 expression and phosphorylation in Tyro42 may provide a helpful target for inhibitory therapies of metastasis in NSCLC. The regulatory significance of PYK2 in NSCLC therefore requires further investigation.

Conflict of interest
None declared.

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References

Up-regulation of proline-rich tyrosine kinase 2 in non-small cell lung cancer


