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Original Article

Prognostic Significance of Phosphorylated Fyn in Patients with Lung Adenocarcinoma after Lung Resection

Shigeto Nishikawa, MD,[1] Toshi Menju, MD, PhD,[1] Koji Takahashi, MD,[1] Ryo Miyata, MD,[1] Makoto Sonobe, MD, PhD,[1] Akihiko Yoshizawa, MD, PhD,[2] and Hiroshi Date, MD, PhD[1]

Purpose: Src family tyrosine kinases, including Fyn, are non-receptor tyrosine kinases that drive malignancy in various kinds of cancers. Fyn has also been suggested to be an effector of epidermal growth factor receptor (EGFR) signaling, and is recognized as a potential therapeutic target. However, little is known about the clinical importance of phosphorylated Fyn (pFyn) in lung adenocarcinoma. The purpose of this study is to examine the prognostic significance of pFyn in this disease.

Methods: A total of 282 lung adenocarcinoma specimens were collected from patients who underwent surgery at our institute. A tissue microarray was assembled from paraffin-embedded tumor blocks. pFyn expression was analyzed through immunostaining of the tissue microarray and each case was classified as positive or negative. The association of clinical information with pFyn expression was analyzed statistically.

Results: pFyn was positive in 107 cases. A pFyn-positive status was significantly associated with male gender, p53 mutant, pathological stage, tumor size, plural invasion, lymphatic invasion, vascular invasion, and differentiation. pFyn positivity was associated with poor relapse-free survival (RFS; hazard ratio [HR]: 2.11, 95% confidence interval [CI]: 1.32–3.42, p <0.01) and poor overall survival (OS; HR: 1.95, 95% CI: 1.17–3.33, p = 0.01).

Conclusion: pFyn expression may affect the prognosis of patients with lung adenocarcinoma after lung resection.

Keywords: phosphorylated Fyn, lung adenocarcinoma, immunohistochemistry, survival analysis

[1]*Department of Thoracic Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Kyoto, Japan*
[2]*Department of Diagnostic Pathology, Kyoto University Hospital, Kyoto, Kyoro, Japan*

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Corresponding author: Toshi Menju, MD, PhD. Department of Thoracic Surgery, Graduate School of Medicine, Kyoto University, Shogoin Kawahara-cho 54, Sakyo-ku Kyoto, Kyoto 606-8507, Japan
Email: toshimnj@kuhp.kyoto-u.ac.jp

Introduction

Lung cancer mortality remains high all over the world.[1)] Recent advances in non-small-cell lung cancer therapy, including tyrosine kinase receptor inhibitors and immune checkpoint inhibitors, have led to dramatic clinical responses[2,3)]; however, these highly potent therapies often end in failure due to drug resistance.[4)] Such drug resistance, whether intrinsic or acquired, is believed to underlie treatment failures in over 90% of patients with metastatic cancers.[5)] ATP-binding cassette (ABC) family transporters and β-tubulin mutations may be involved in drug resistance,[6,7)] but their roles as essential

factors remain uncertain.[8] Establishment of more effective anticancer therapies will require determination of the main resistance pathways.

We have reported ABCB1 overexpression, activation of the focal adhesion pathway, and its availability for inhibition in vinorelbine-resistant cells.[9] Focal adhesion pathways, particularly integrins and Src family kinases (SFKs), play important roles in cancer cell survival, invasion, proliferation, and drug resistance.[10,11] SFKs, including Fyn, are non-receptor tyrosine kinases that drive malignancy in various cancers.[12,13] Fyn has also been suggested to be an effector of epidermal growth factor receptor (EGFR) signaling,[14,15] and it is recognized as a potential therapeutic target.[11,16,17] However, little is known about the clinical importance of phosphorylated Fyn (pFyn) in lung adenocarcinoma. Therefore, the purpose of this study is to examine the prognostic significance of pFyn in this disease.

Methods

Samples from patients

A total of 282 lung adenocarcinoma specimens were collected from patients who underwent surgery at our institute from January 2001 to December 2007. Among these specimens, 54 were excluded from analysis because the specimen on the tissue microarray was inappropriate for evaluation (15 cases), cDNA was not available (20 cases), or the pathological stage was IIIB or IV (19 cases). The follow-up time ranged from 1 to 129 months (median 63 months). Data, including relapse-free survival (RFS) times, overall survival (OS) times, and outcomes, were available for all patients. The Kyoto University Graduate School and Faculty of Medicine Ethics Committee approved this study (approved number: G0028-7, R1706). We obtained written informed consent for tumor tissue usage from all patients. A pathologist (A.Y.) reviewed all tumors and confirmed predominant tumor subtypes, node status, and local lymph-vascular involvements. Based on the TNM classification of the International Union Against Cancer, 7th edition, all tumors were restaged.

Tissue microarrays

Pathologists in the Department of Diagnostic Pathology in our institute decided the most representative areas of the tumors based on the morphology of the individual hematoxylin and eosin-stained slides. According to the approach described by Kononen et al.,[18] pathologists assembled tissue microarrays from paraffin-embedded tumor blocks. In all, 48 tissue cores of 2 mm diameter were arrayed in each paraffin block, which included non-neoplastic lung tissue cores from selected patients as controls.

Immunohistochemical analysis

Using the standard avidin-biotin-peroxidase complex method, we performed immunohistochemical staining for pFyn, E-cadherin, and vimentin with rabbit anti-human pFyn polyclonal antibodies (AP0510, dilution 1:400, ABclonal, York, UK), mouse anti-human E-cadherin monoclonal antibody (36B5, dilution 1:300, Leica Biosystems, Newcastle upon Tyne, UK), and mouse anti-human vimentin monoclonal antibody (SRL33, dilution 1:300, Leica Biosystems). 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan) was used for visualization with hematoxylin counterstaining. Four investigators (SN, TM, KT, and RM) independently and blindly scored the immunostained sections. After that, we re-evaluated the assessment to eliminate the dissidence. After immunostaining, expression of E-cadherin and vimentin was categorized as positive and negative as previously described.[19] Epithelial-mesenchymal transition (EMT) status was classified into three categories[19]: full EMT (E-cadherin negative, vimentin positive), partial EMT (both E-cadherin and vimentin positive, or both negative), and null EMT (E-cadherin positive, vimentin negative). Expression of pFyn in the nucleus was also examined and categorized as positive or negative.

PCR and DNA sequencing

Using an RNeasy Plus mini kit (QIAGEN, Valencia, CA, USA), total RNA was extracted from tumor samples that had been frozen and stocked after resection. Total RNA was reverse transcribed to cDNA using a Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Life Sciences, Pittsburgh, PA, USA). For PCR amplification, each cDNA was diluted to 10 ng/μL. PCR conditions were as follows: p53 exon 4 forward: 5′-CCC AAG CAA TGG ATG ATT TG-3′; p53 exon 10 reverse: 5′-AGC CTG GGC ATC CTT GAG-3′. The PCR assay was carried out in a 15 μL volume that contained 15 ng of cDNA and 1 unit of Taq PCR Master Mix Kit (QIAGEN). Each PCR reaction was started at 95°C for 5 min, and then cDNA was amplified for 40 cycles at 95°C for 30 s, 54.7°C for 30 s, and 72°C for 90 s, with a final extension

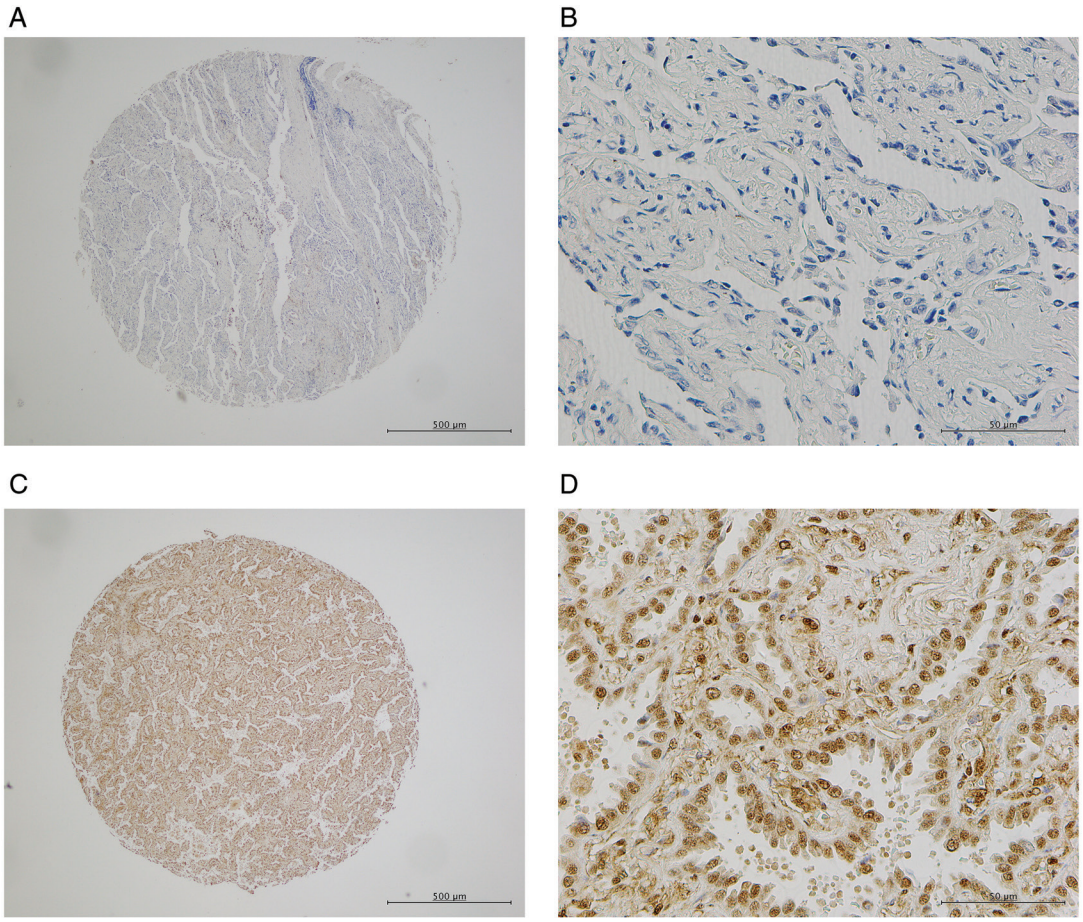

Fig. 1 Immunohistochemical staining of pFyn in lung adenocarcinoma. Representative images of negative expression (**A**: ×40, **B**: ×400) and positive expression (**C**: ×40, and **D**: ×400). pFyn: phosphorylated Fyn

time of 7 min at 72°C. Each amplicon was purified using a QIAquick Gel Extraction Kit (QIAGEN) after agarose gel electrophoresis. Purified PCR products were sequenced in forward and reverse directions using a 3130xl Genetic Analyzer (Thermo Fisher Scientific K.K.). p53 mutations were detected in exons 5 through 8, as in previous reports.[20,21]

Statistical analyses

Baseline characteristics were compared between pFyn-positive and pFyn-negative groups using a t-test for continuous variables and a chi-square test for categorical variables. Time-to-event curves for RFS and OS were estimated using the Kaplan–Meier method, and differences in time-to-event curves were evaluated by log-rank test, with HRs estimated using a Cox regression model. A p value of <0.05 was considered significant. Statistical analyses were conducted using JMP Pro 13 (SAS Institute, Cary, NC, USA).

Results

pFyn expression in resected lung adenocarcinoma

Expression of pFyn, E-cadherin, and vimentin was analyzed using immunohistochemical analysis of the tissue microarray. pFyn-positive signaling was mainly located in nuclei of tumor cells and it was stained homogeneously in each positive case, as shown in **Fig. 1**. pFyn was positive in 107 cases (46.9%). As we reported previously, E-cadherin and vimentin positive signaling was located in cytoplasm of tumor cells.[19] E-cadherin was positive in 123 cases (53.9%) and vimentin was positive in 47 cases (20.6%) in this study.

The clinicopathological characteristics of the 228 patients are summarized in **Table 1**. A pFyn-positive status was significantly related to several clinicopathological features (**Fig. 2**): male gender (risk ratio [RR] = 1.84, 95% confidence interval [CI]: 1.08–3.11, p = 0.02), p53 mutant (RR = 2.01, 95% CI: 1.08–3.72, p = 0.03), advanced

Table 1 Comparison of clinical characteristics of pFyn-positive and pFyn-negative cases

Characteristics	pFYN positive (N = 107)	pFYN negative (N = 121)	p value
Age, years, mean±SD	66.3 ± 0.93	66.3 ± 0.88	0.51
Male, N (%)	63 (58.9)	53 (43.8)	0.02
Smoking status, N (%)			0.27
Never	43 (40.2)	57 (47.1)	
Former	34 (31.8)	27 (22.3)	
Current	30 (28.0)	37 (30.6)	
p53 mutation, N (%)	33 (30.8)	22 (18.2)	0.03
EGFR mutation, N (%)	55 (49.6)	56 (46.3)	0.44
p-Stage, N (%)			<0.01
IA	41 (38.3)	73 (60.3)	
IB	38 (35.5)	29 (24.0)	
IIA	10 (9.4)	12 (9.9)	
IIB	2 (1.9)	2 (1.7)	
IIIA	16 (15.0)	5 (4.1)	
E-cadherin positive, N (%)	56 (52.3)	67 (55.4)	0.65
Vimentin positive, N (%)	22 (20.6)	25 (20.7)	0.99
EMT status, N (%)			0.35
Full	19 (17.8)	15 (12.4)	
Partial	35 (32.7)	49 (40.5)	
Null	53 (49.5)	57 (47.1)	
Tumor size, mm, mean±SD	28.1 ± 1.28	23.8 ± 1.20	0.01
Plural invasion, positive, N (%)	32 (29.9)	17 (14.0)	<0.01
Lymphatic invasion, positive, N (%)	27 (25.2)	17 (14.1)	0.01
Vascular invasion, positive, N (%)	33 (30.8)	19 (15.8)	0.01
Differentiation, N (%)			0.01
Well differentiation	12 (11.2)	32 (26.5)	
Moderate differentiation	44 (41.1)	47 (38.8)	
Poor differentiation	51 (47.7)	42 (34.7)	

EGFR: epidermal growth factor receptor; EMT: Epithelial-mesenchymal transition; pFyn: phosphorylated Fyn

pathological stage (stage III vs. stage I, RR = 4.13, 95% CI: 1.45–11.76, p = 0.01, stage III vs. stage II, RR = 3.73, 95% CI: 1.05–13.24, p = 0.04), tumor size (≥20 mm, RR = 2.26, 95% CI: 1.28–4.01, p <0.01), pleural invasion (positive, RR = 2.61, 95% CI: 1.35–5.04, p <0.01), lymphatic invasion (positive, RR = 2.06, 95% CI: 1.05–4.05, p = 0.03), vascular invasion (positive, RR = 2.39, 95% CI: 1.26–4.54, p = 0.01), and differentiation (moderate vs. well, RR = 2.50, 95% CI: 1.14–5.45, p = 0.02, poor vs. well, RR = 3.23, 95% CI: 1.48–7.06, p <0.01).

pFyn expression showed no significant association with E-cadherin or vimentin expression. The fully activated EMT rate was slightly higher in the pFyn-positive group, but the difference was not significant. There was also no significant association of pFyn with age, EGFR mutation, or smoking history.

Association of pFyn positivity with prognosis

The impact of a pFyn-positive status on RFS and OS is shown in **Fig. 3**. pFyn positivity was significantly associated with poor RFS (HR: 2.11, 95% CI: 1.32–3.42, p <0.01) (**Table 2**). The estimated median RFS was 96 months in the pFyn-positive group, while that in the pFyn-negative group was not reached. RFS at 60 months was estimated to be 58.0% and 78.4% in the respective groups. pFyn positivity was also significantly associated with poor OS (HR: 1.95, 95% CI: 1.17–3.33, p = 0.01) (**Table 2**). The estimated median OS was 109 months in the pFyn-positive group, and was not reached in the pFyn-negative group. OS at 60 months was estimated to be 71.1% and 83.5% in the respective groups.

Discussion

Proteins in the focal adhesion pathway, including integrins and SFKs, have crucial roles in cancer malignancy.[10,11] We have shown that pFyn expression is upregulated in parallel with ABCB1 expression, which impairs drug sensitivity,[9] but little is known about the clinical importance of Fyn expression in lung adenocarcinoma. We note that in

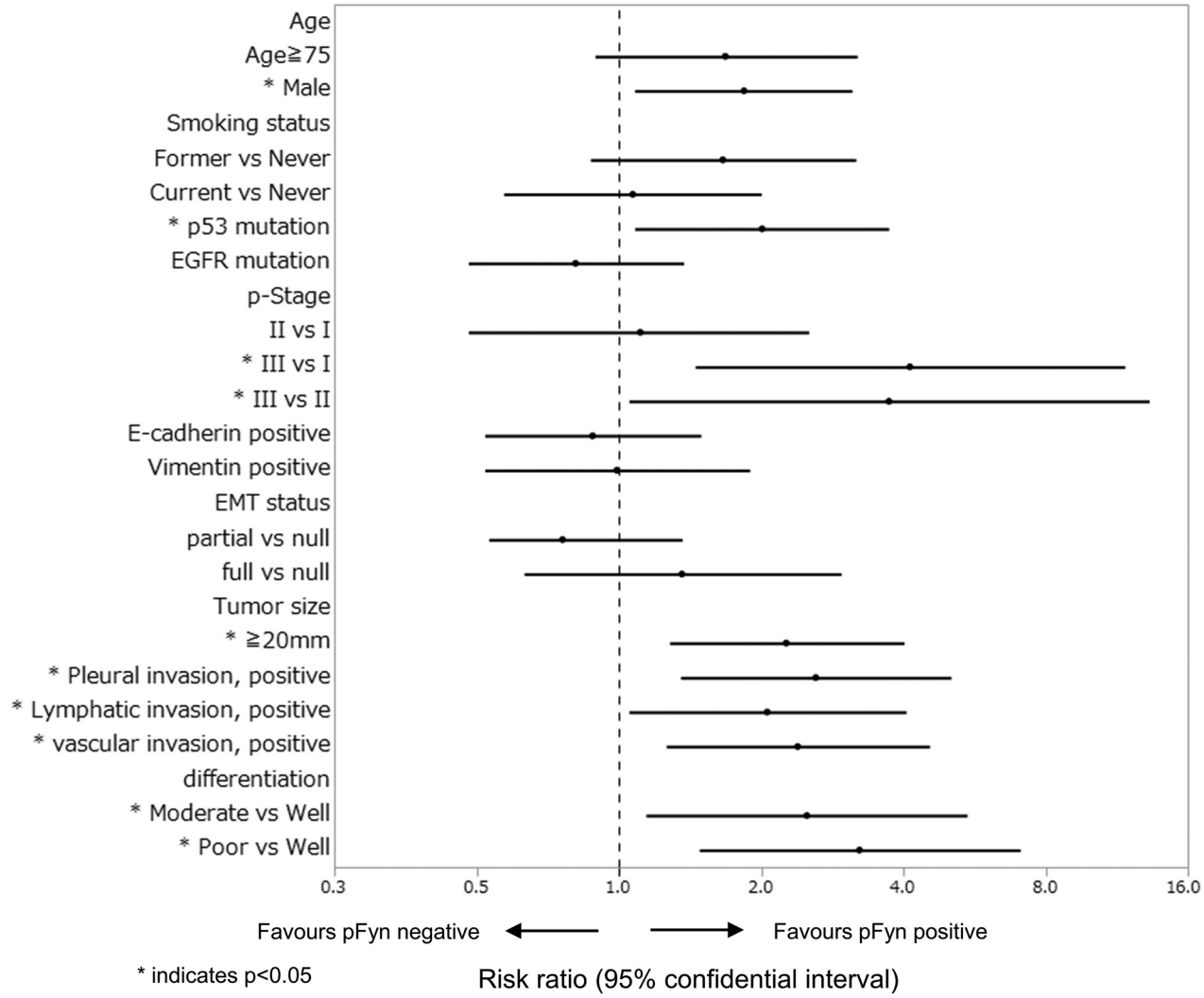

Fig. 2 Forest plot of risk for a pFyn status based on baseline characteristics. pFyn: phosphorylated Fyn; EGFR: epidermal growth factor receptor; EMT: epithelial-mesenchymal transition

this study we used an antibody that is specific for pFyn, compared to other SFK, based on our previous study.[9] It will be important to examine the differences among SFKs, but these experiments were beyond the purpose of this study. The most important result in this study is the novel finding of the prognostic significance of pFyn in adenocarcinoma after lung resection.

We found significant associations of clinicopathological characteristics with pFyn expression. pFyn was significantly associated with representative malignant features of cancer, such as advanced pathological stage, tumor size, local invasive factors, and differentiation. pFyn was also associated with poor RFS and OS. These results are consistent with previous reports on SFKs.[12,13] In contrast, pFyn expression was not related to EGFR mutation, despite previous reports suggesting that Fyn is an effector of EGFR signaling.[14,15] We cannot draw a definite conclusion regarding this discrepancy, and further investigation such as measuring the activation level of EGFR is needed. Based on our previous study,[19] we examined the association of EMT status with pFyn expression; however, no significant association was observed. We speculate that pathways that overexpress pFyn do not contribute to EMT activation.

There are several limitations to the study, including the small number of cases and performance of a retrospective single institute analysis. Propensity score matching is often used to adjust for potential bias that may influence prognosis; however, we could not use this method because our dataset was not large enough to adjust for all pFyn-related clinicopathological characteristics. We were also unable to investigate the response

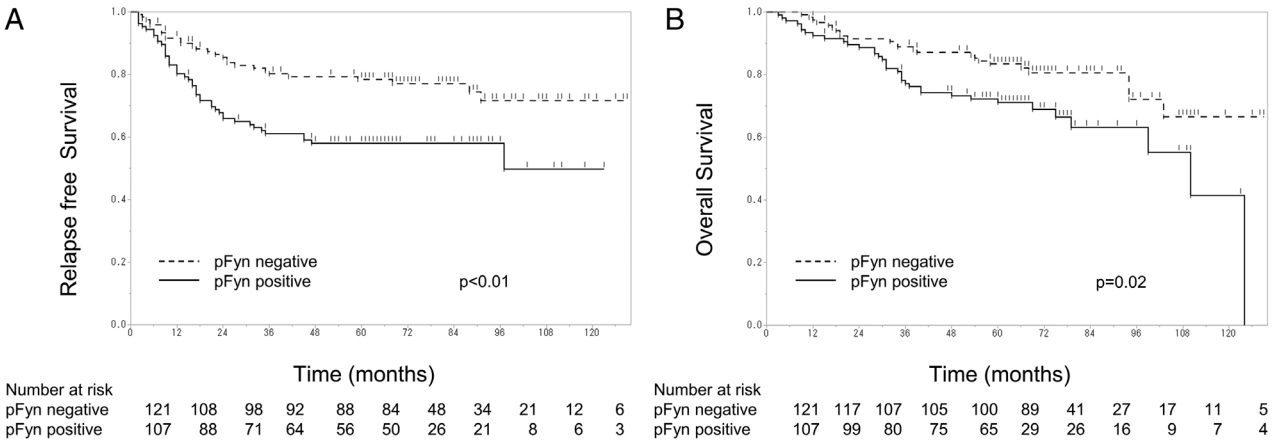

Fig. 3 Analyses of associations of a pFyn-positive status with prognosis. Kaplan–Meier relapse-free (**A**) and overall (**B**) survival curves. Numbers at risk are listed. pFyn: phosphorylated Fyn

Table 2 Relapse-free and overall survival in pFyn-positive and pFyn-negative cases

	pFyn positive	pFyn Negative	HR (95% CI)[a]	p value	Figure
Relapse-free survival	96	Not estimated	2.11 (1.32–3.42)	<0.01	3A
Overall survival	109	Not estimated	1.95 (1.17–3.33)	0.01	3B

[a]Hazard ratio (95% CI).

CI: confidence interval; pFyn: phosphorylated Fyn

against adjuvant chemotherapy because only 47 patients (28 pFyn-positive, 19 pFyn-negative) were in an advanced stage (IIA, IIB, and IIIA). Therefore, we focused only on evaluating the relationship between clinicopathological characteristics and pFyn.

Additional therapy against pFyn may improve the prognosis of patients with lung cancer, and this may be an interesting research area. Accumulation of in vitro and in vivo data will be required to determine how pFyn influences cancer malignancy. Further independent validation is required, but our results should contribute to development of new anticancer therapies targeting pFyn.

In this study, pFyn expression investigated by tissue microarray and immunohistochemistry was highly associated with malignant features of lung cancer. These results suggest that pFyn expression may affect the prognosis of patients with lung adenocarcinoma after lung resection.

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Notation of Prior Presentation

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Disclosure Statement

The authors have no conflict of interest to declare

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