Activation of Janus kinase 1 confers poor prognosis in patients with non-small cell lung cancer

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Abstract. The activation of Janus kinase 1 (JAK1) has been reported to occur in non-small cell lung cancer (NSCLC), activating the JAK/signal transducers and activators of transcription cascade. However, the association between JAK1 activation and the prognostic value in NSCLC remains unclear. The present study initially investigated the association between expression of the activated form of JAK1 (p-JAK1) and prognosis in patients with NSCLC. A cohort of 142 resected primary NSCLC tissue samples, including 74 adenocarcinoma (ADCC) and 68 squamous cell carcinoma samples, were analyzed. p-JAK1 expression status was determined by immunohistochemistry. Evaluation of epidermal growth factor receptor (EGFR) gene amplification by fluorescence *in situ* hybridization was subsequently performed in 74 ADCC samples. The prognostic significance of p-JAK1 expression and EGFR gene amplification were evaluated with univariate and multivariate survival analyses. Compared with normal lung tissue, p-JAK1 expression level was significantly increased in NSCLC (P<0.001). Positive p-JAK1 expression indicated a poor prognosis, particularly for patients in early stages (stage I/II, including tumor size <3 cm, Lymph node invasion N0/1; all P<0.05). p-JAK1 expression was an independent predictor of a poor prognosis (P=0.022). The overall survival time for patients with positive p-JAK1 expression and EGFR-amplified tumors was significantly shortened compared with patients with tumors negative for one or both features (both features present vs. neither feature present, P<0.001). The results provided clinical evidence that the activation of JAK1 was an independent prognostic factor, particularly in early stage NSCLC. The combination of EGFR gene amplification and p-JAK1 expression may be a novel target for the selection of individual therapy strategies and predicting the effects of therapy for NSCLC.

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Introduction

Lung cancer is one of the leading causes of cancer-associated mortality, accounting for 27% (including 26% for females, and 28% for males), and non-small cell lung cancer (NSCLC) accounts for 80-85% of all lung cancer-associated mortalities (1,2). Despite advances in the understanding of the molecular mechanisms of lung cancer and the development of novel chemotherapeutic agents, the 5-year survival rates for lung and bronchus cancer remained <18% from 2004 to 2010 (2). A number of studies have focused on progressing the understanding of oncogenic kinase signaling pathways, which has provided targets for developing effective therapeutic strategies in order to improve clinical outcomes (3).

One of the potential candidates for therapy is the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. JAK/STAT is one of the pleiotropic cascades that may transduce a multitude of signals for development and homeostasis in animals, from humans to flies (4). JAK family members have been reported to be dysregulated in malignant tumors, including in colorectal, prostate and myeloproliferative cancer (5-8). The mammalian JAK family includes four members: JAK1, JAK2, JAK3 and Tyk2. All JAKs exhibit broad patterns of expression with the exception of JAK3, which is restricted to leukocytes (9).

JAK1 binds to various cytokines non-covalently to mediate cell proliferation and differentiation (10). JAK1 knockout mice die perinatally (9). JAK1 mutation has been reported in hepatocellular carcinoma, acute lymphoblastic

leukemia, lung and gastric cancer (10,11). Phosphorylated (p)-JAK1, the active form of JAK1, mediates the phosphorylation of receptors and the major substrates for JAK family members, STATs (4). For example, p-JAK1 expression was detected in primary esophageal squamous cell carcinoma and not in normal esophageal squamous cells. p-JAK1 expression was associated with a reduced overall survival time (12). Additionally, a previous study by the present authors demonstrated that JAK1 expression was significantly increased in NSCLC clinical samples compared with normal samples (P>0.001), while p-JAK1 (Tyk 1022) showed trends of positive expression, though these did not reach statistical significance (P=0.055), potentially owing to a small sample size (13). This indicated that JAK1 activation was abnormal in NSCLC.

Lung adenocarcinoma (ADCC) is the most common histological subtype of NSCLC. Patients with ADCC and a high epidermal growth factor receptor (EGFR) copy number can be treated with EGFR-tyrosine kinase inhibitors (14,15). EGFR gene amplification has also been reported to be associated with prognosis of lung cancer, though there is some controversy in this regard (16,17). However, the combination of EGFR gene amplification and JAK1 activation for predicting cancer prognosis has not been extensively studied. This has incited the present study, which will investigate associations between JAK1 activation, EGFR gene amplification and survival status in patients with NSCLC.

Materials and methods

Tissue collection. The study cohort consisted of 142 patients (40 female and 102 male) with a median age of 63 years (range, 20-84 years). A total of 142 paraffin-embedded resected primary NSCLC samples were analyzed from the archives of the Pathology Department at Sichuan Provincial People's Hospital (Chengdu, China) from December 2004 to February 2007, including 74 cases of ADCC and 68 cases of squamous cell carcinoma (SqCC) A total of 142 adjacent normal pulmonary tissue specimens were also resected in the same tissue blocks. Staging was performed according to the International Union Against Cancer's tumor-node-metastasis system (18). Differentiation and histological type were scored according to the World Health Organization classification for NSCLC (19). None of the patients had received neoadjuvant therapy prior to surgical resection. Following the surgery, the patients underwent standard therapy procedure, according to the National Comprehensive Cancer Network Clinical Practice Guideline for Oncology, NSCLC, 2004 (20). Informed consent was obtained from all individuals included in the present study. Institutional review board approval for the study was obtained from Sichuan Provincial People's Hospital.

Immunohistochemical staining (IHC). IHC staining was performed as previously described (3). Briefly, the 4-μm sections underwent deparaffinization, hydration and endogenous peroxide blocking. Antigen retrieval was performed by heating at 95˚C for 30 min in Tris/ethylenediaminetetraacetic acid retrieval solution. The sections were blocked with 3% bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and incubated with primary p-JAK1 antibody (cat. no. 11149, 1:100 dilution; Signalway Antibody LLC., College Park, MY, ML, USA) overnight at 4˚C. The membranes were subsequently incubated with enough secondary peroxidase-labeled polymer-conjugated goat anti-rabbit antibody to cover the specimen (cat. no. K4007; undiluted; EnVsion; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), according to the manufacturer's protocol, for 30 min at room temperature. For staining, 3,3-daiminobenzidine chromogen was applied. Finally, the sections were counterstained with hematoxylin for 3 min at room temperature, fixed and mounted.

Table I. p-JAK1 expression in NSCLC and adjacent normal tissues.

Parameter	p-JAK1$^+$, n (%)	p-JAK1$^-$, n (%)	P-value[a]
NSCLC tissue	56 (39.4)	86 (60.6)	<0.001
Normal control	11 (7.7)	131 (92.3)	

[a]By Perason's χ^2 test. p-JAK1, phosphorylated Janus kinase 1; NSCLC, non-small cell lung carcinoma.

Scoring for immunohistochemical staining. Scoring for immunochemical staining was evaluated as previously described (3) by two independent pathologists. Briefly, the expression of p-JAK1 was assessed semi-quantitatively on the basis of criteria that accounted for the fraction and intensity of immunostaining of the tumor cells. The fraction score was defined as: 0 (0%), 1 (<20%), 2 (20-50%) and 3 (>50%). The intensity of p-JAK1 staining was scored as 0 (no appreciable staining), 1 (barely detectable staining), 2 (readily identifiable brown staining) and 3 (dark brown staining). A total score was calculated by multiplying the fraction and the intensity score. A tumor sample was considered positive if the score was ≥4, and negative otherwise.

Determination of EGFR gene amplification by fluorescence in-situ hybridization (FISH). EGFR FISH analysis was performed with the dual-color EGFR SpectrumOrange/CEP7 SpectrumGreen probe and paraffin pretreatment reagent kit (both from Vysis; cat. nos. 05J48-001 and 32-801210, respectively; Abbott Laboratories, Chicago, IL, USA) as previously described (21). Briefly, the paraffin sections were deparaffinized, dehydrated and digested with protease K. The slides were denatured by heating to 75˚C for 5 min and dehydrated in ethanol. The probes were denatured for 5 min at 75˚C prior to hybridization. Each slide was hybridized at 37˚C overnight and washed in 2X saline-sodium citrate buffer/0.3% NP40 at 72˚C for 2 min. The nuclei were counterstained with DAPI/antifade 1 (Vysis; Abbott Laboratories, Chicago, IL, USA). A minimum of 100 non-overlapping tumor cell nuclei were scored in each case, according to the University of Colorado Cancer Center criteria (22). The BX-51/Genus FISH Imaging system (DP71/70/DP30BW software, DP-BSW Ver.03.02) was used for imaging (Olympus, Tokyo, Japan).

Table II. Clinical characteristics and prognosis of patients with non-small cell lung carcinoma.

Parameter	Patients, n	Median survival time, range (months)	P-value[a]
Age			0.005
<60 years	56	63 (3-94)	
≥60 years	86	60 (1-96)	
Gender			0.561
Female	40	63 (3-96)	
Male	102	61 (1-94)	
Histological type			0.959
Adenocarcinoma	74	61 (3-96)	
Squamous cell carcinoma	68	62.5 (1-94)	
Tumor size			0.446
<3 cm	59	61.5 (6-96)	
≥3 cm	83	60 (1-94)	
Lymph node invasion			<0.001
N0/1	122	63 (1-96)	
N2/3	20	39 (3-67)	
Distant metastasis			0.973
M0	138	61 (1-96)	
M1	4	57.5 (41-85)	
TNM stage			0.001
I/II	92	62.5 (1-96)	
III/IV	50	51.5 (3-89)	

[a]By log-rank test. TNM, tumor-node-metastasis staging system.

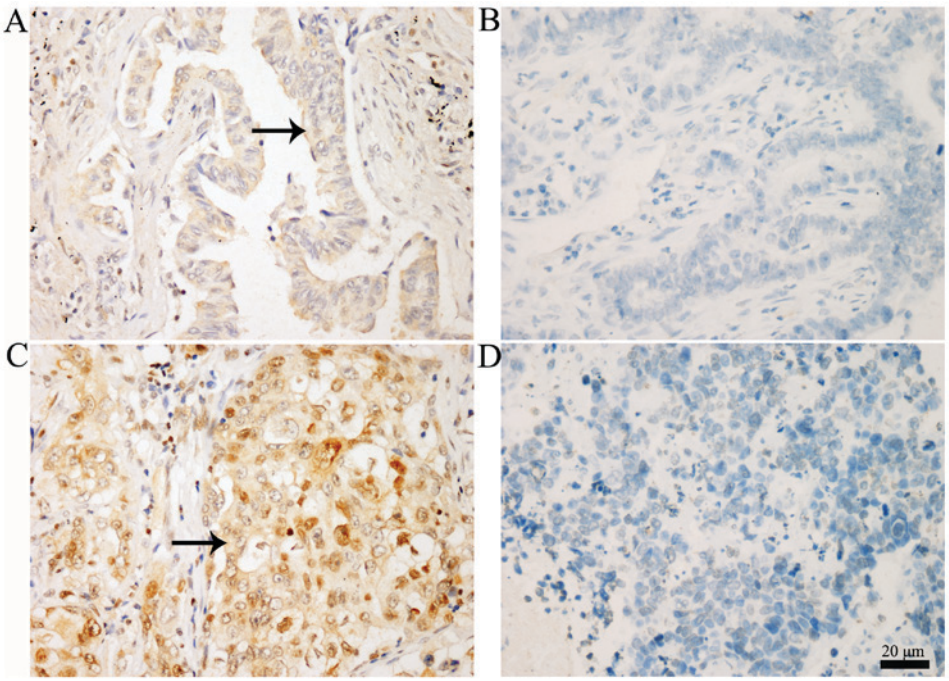

Figure 1. Representative images of p-JAK1 positive and negative expression in non-small cell lung carcinoma. Expression of p-JAK1 was observed in the cytoplasm and nucleus, as indicated by the arrows. (A) Positive and (B) negative p-JAK1 expression in adenocarcinoma; (C) positive and (D) negative p-JAK1 expression in squamous cell carcinoma. Magnification, x400. p-JAK1, phosphorylated Janus kinase 1.

Statistical analysis. The association between clinical characteristics and p-JAK1 expression was determined using Pearson's χ^2 test. When one of the expected values in a 2x2 table was <5, Fisher's exact test would be used. The

Table III. p-JAK1 expression and clinical characteristics in patients with non-small cell lung cancer.

Characteristic	p-JAK1$^+$	p-JAK1$^-$	P-value
Age			0.867[a]
<60	22	35	
≥60	34	51	
Gender			0.290[a]
Female	13	27	
Male	43	59	
Histological type			0.014[a]
Adenocarcinoma	22	52	
Squamous cell carcinoma	34	34	
Tumor size			0.029[a]
<3 cm	17	42	
≥3 cm	39	44	
Lymph node invasion			0.297[a]
N0/1	46	76	
N2/3	10	10	
Distant metastasis			0.300[b]
M0	53	85	
M1	3	1	
TNM stage			0.009[a]
I/II	29	63	
III/IV	27	23	

[a]By Perason's χ^2 test; [b]by Fisher's exact test. p-JAK1, phosphorylated Janus kinase 1; TNM, tumor-node-metastasis staging system.

Table IV. Survival distributions between groups separated by clinical characteristics and p-JAK1 expression.

Comparisons	Hazard ratio	Confidence interval (95%)	P-value[a]
NSCLC			
p-JAK1$^+$ vs. p-JAK1$^-$	1.929	1.100-3.382	0.022
N0/1 vs. N2/3[b]	4.256	2.253-8.037	<0.001
ADCC			
p-JAK1$^+$ vs. p-JAK1$^-$	2.285	1.014-5.147	0.046
TNM stage I/II vs. III/IV	3.551	1.573-8.017	0.002
SqCC			
N0/1 vs. N2/3[b]	4.110	1.481-11.407	0.007

[a]By Cox regression multivariate analysis; [b]Lymph node invasion. ADCC, adenocarcinoma; NSCLC, non-small cell lung carcinoma; p-JAK1, phosphorylated Janus kinase 1; TNM, tumor-node-metastasis staging system; SqCC, squamous cell carcinoma.

Kaplan-Meier method was used for the univariate analysis of survival time between groups based on clinical characteristics and p-JAK1 expression. Multivariate analysis was performed using Cox regression model analysis. Only markers that were significant predictors in univariate analysis were included in the multivariate analysis. All tests were two-sided and P<0.05 was considered to indicate a statistically significant difference. SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for these analyses.

Results

JAK1 expression in non-small cell lung cancer tissues. Representative IHC staining images of p-JAK1 expression in NSCLC and adjacent normal lung tissues are displayed in Fig. 1. Positive p-JAK1 expression was observed in the cytoplasm and nucleus, as indicated by arrows. In the NSCLC tissues, 56/142 cases (39.4%) exhibited positive p-JAK1 expression, whereas 11/142 cases (7.7%) were positive in the

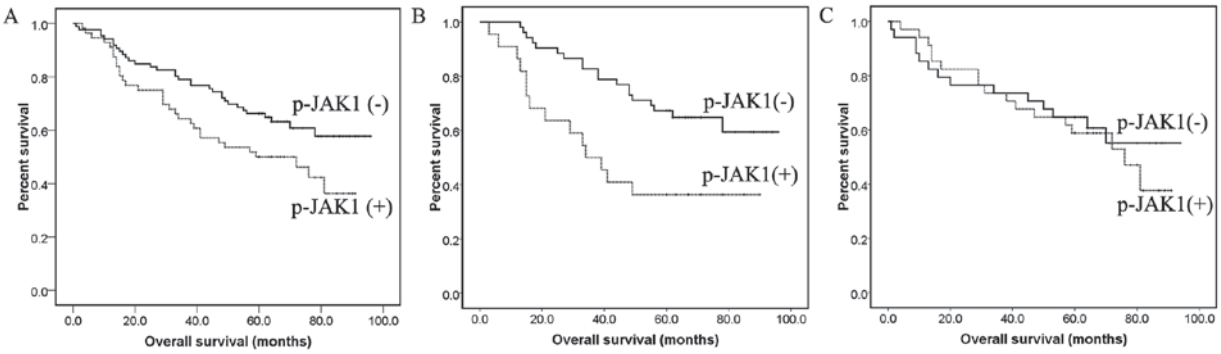

Figure 2. Association between p-JAK1 expression status and survival time in NSCLC. Kaplan-Meier curves compare the overall survival time of patients with positive (dotted line) or negative (solid line) p-JAK1 expression. Kaplan-Meier curves for (A) all types of NSCLC, (B) adenocarcinoma and (C) squamous cell carcinoma subtypes are illustrated. p-JAK1, phosphorylated Janus kinase 1; NSCLC, non-small cell lung carcinoma.

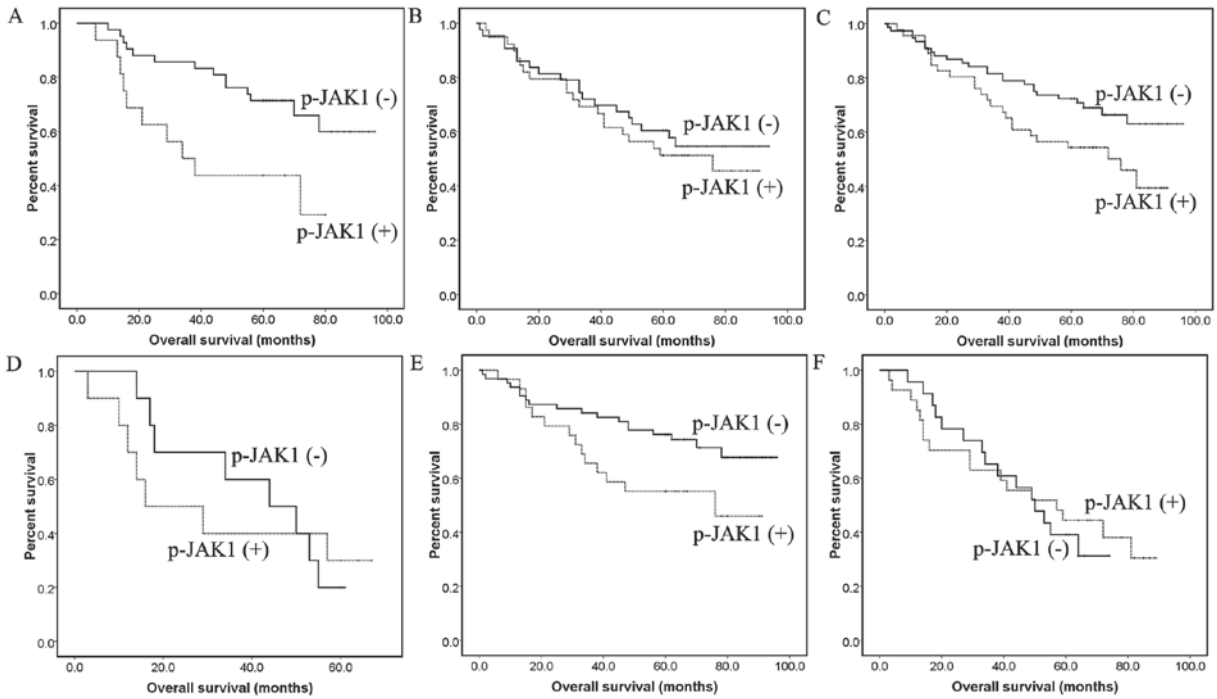

Figure 3. Association between p-JAK1 expression status and survival time in early and late stage NSCLC. Kaplan-Meier curves for patient survival are stratified by p-JAK1 positive (dotted line) and negative expression (solid line) in (A) Tumor size <3 cm (B) Tumor size ≥3 cm, (C) N0/1 (D) N2/3 (E) stage I/II and (F) stage III/IV groups, respectively. p-JAK1, phosphorylated Janus kinase 1; NSCLC, non-small cell lung carcinoma.

adjacent normal tissues (Table I). The difference was highly significant (P<0.001).

Association between clinical characteristics and prognosis in patients with NSCLC. As listed in Table II, follow-up information was available for all patients. Survival time differed significantly by age (>60 vs. ≤60), lymph node invasion (N0/1 vs. N2/3) and stage (I/II vs. III/IV; P=0.005, P<0.001 and P=0.001, respectively). Gender, tumor size and histological type were not significantly associated with survival time.

Association between clinical characteristics and p-JAK1 expression. Pearson's χ^2 or Fisher's exact test were performed to assess the significance of associations between clinical characteristics and p-JAK1 expression. As shown in Table III, p-JAK1 expression was associated with histological type, as 29.7% of ADCC samples and 50.0% of SqCC samples were p-JAK1$^+$ (P=0.014). p-JAK1 expression status was also associated with tumor size and stage (P=0.021 and P=0.009, respectively). Other clinical characteristics, including age, gender, lymph node invasion and distant metastasis, were not associated with p-JAK1 expression status (P>0.05).

Survival status and p-JAK1 expression. Positive p-JAK1 expression was associated with a poor prognosis in NSCLC (P=0.039; Fig. 2A-C). However when divided by histological type, the difference was only statistically significant in ADCC samples (P=0.007) and not in SqCC (P=0.612). Subjects with p-JAK1 expression demonstrated reduced survival time in early stage NSCLC, including patients with tumor size <3 cm, N0/1 and stage I/II (P=0.016, P=0.034 and P=0.048, respectively; Fig. 3A-F). The difference in survival time in patients

Table V. Effects of p-JAK1 expression and EGFR gene duplication status on the prognosis of patients with lung adenocarcinoma, as determined by Kaplan-Meier analysis.

Status	Patients, n	Median survival time (months)	P-value[a]
p-JAK1$^+$/EGFR$^+$	7	16 (3-60)	
p-JAK1$^-$/EGFR$^-$	30	61.5 (15-96)	<0.001
p-JAK1$^-$/EGFR$^+$	22	62 (13-94)	0.004
p-JAK1$^+$/EGFR$^-$	15	49 (12-90)	0.052

[a]By log-rank test, compared with p-JAK1+/EGFR+ patients. p-JAK1, phosphorylated Janus kinase 1; EGFR, epidermal growth factor receptor.

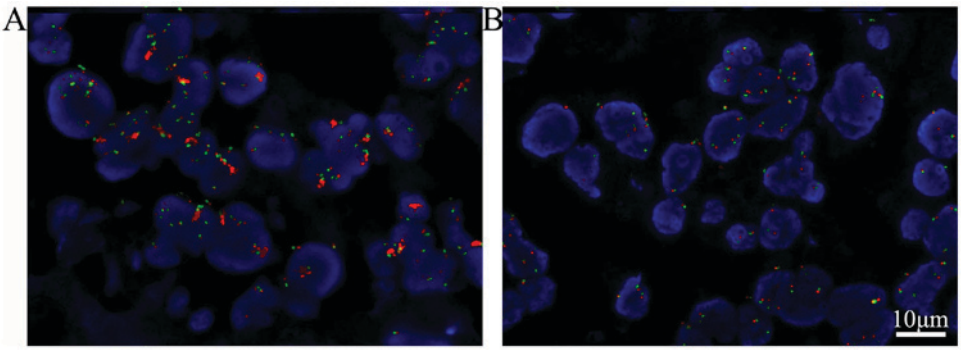

Figure 4. Determination of EGFR gene amplification by FISH. Evaluation of EGFR gene copy number by FISH analysis was performed using an EGFR (orange)/CEP 7 (green) probe with DAPI nuclear counterstaining. Representative examples are included of (A) a sample featuring EGFR gene duplication and (B) a sample with a normal number of EGFR genes. Magnification, x400. EGFR, epidermal growth factor receptor; FISH, fluorescence *in situ* hybridization.

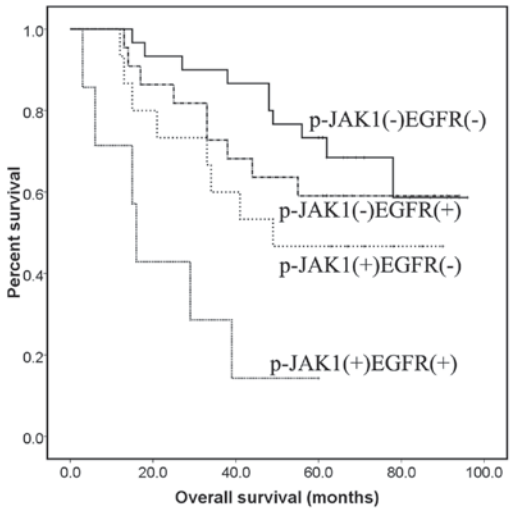

Figure 5. Association between the combination of p-JAK1 expression and EGFR gene duplication, and survival time in patients with lung adenocarcinoma. Kaplan-Meier curves for patient survival are stratified by p-JAK1 positive or negative expression with EGFR duplication positive or negative status. Solid line, p-JAK1$^-$/EGFR$^-$ expression; dashed line, p-JAK1$^-$/EGFR$^+$; half-dotted line, p-JAK1$^+$/EGFR$^-$, dotted line, p-JAK1$^+$/EGFR$^+$. p-JAK1, phosphorylated Janus kinase 1; EGFR, epidermal growth factor receptor.

with late stage disease, including tumor size ≥3 cm, N2/3 or stage III/IV was not significant.

Survival status and p-JAK1 and EGFR expression in ADCC. To investigate whether associations between JAK1 activation, EGFR gene amplification and prognosis in patients with ADCC were significant, EGFR status was examined with FISH analysis, and univariate survival analyses were performed for the 74 ADCC subjects (Table V). EGFR gene amplification was determined by FISH (Fig. 4), 29/74 ADCC cases exhibited EGFR gene amplification (designated as EGFR$^+$). As shown in Table V, survival time for patients with p-JAK1$^+$/EGFR$^+$ was significantly reduced compared with those with p-JAK1$^-$/EGFR$^-$ or p-JAK1$^-$/EGFR$^+$ combinations (P<0.001 and P=0.004, respectively), but not the p-JAK1$^+$/EGFR$^-$ combination (P=0.052; Fig. 5). The results indicated that the EGFR amplification and p-JAK1$^+$ combination may be a novel target to inform the selection of individual therapy strategies and for predicting the effect of therapy in ADCC.

Discussion

Phosphorylation of JAK1 is important for the activation of JAK/STAT pathways and their downstream cascades (10). In the present study, it was identified that the activation of JAK1 was associated with a poorer prognosis in NSCLC (P=0.039), particularly in ADCC (P=0.007). p-JAK1 was identified as an independent predictor for poor prognosis (P=0.022). Overall survival time for patients with p-JAK1$^+$ and EGFR gene duplication was significantly reduced compared with patients with one or neither trait (P=0.001).

A previous study by the present authors and studies by others have indicated that inhibition of JAK signaling exhibits anticancer and anti-angiogenetic effects in human cancer

lines and xenograft tumors, including lung cancer (13,23-25). These results support the hypothesis that JAK1 activation is important for tumorigenesis in NSCLC. In the present study, when subdivided by histotype, differences in prognosis were identified as statistically significant. Positive p-JAK1 was an independent predictor for decreased survival times only for patients with ADCC, not SqCC. This finding suggests a specific role for JAK1 in lung ADCC. In several lung ADCC cell lines, JAK family inhibitor AZD1480 has demonstrated to be able to potently block STAT3 signaling and oncogenesis (24), which supports the results of the present study to some extent. However, the research on the effect of p-JAK1 expression on prognosis in lung SqCC has been limited. You *et al* (12) reported that JAK/STAT pathway activations were associated with shorter survival time in esophageal SqCC cases. As only 68 cases of SqCC patients were recruited to the present study, further evaluation should be performed using a larger amount of cases.

In the present study, it was further identified that tumor size <3 cm, N0/1 and stage I/II patients with positive p-JAK1 expression had shorter survival time (all $P<0.05$), whereas the difference was not significant for patients in advanced stages (Fig. 3). This finding indicated that the activation of JAK1 may be an early event in the tumorigenesis of NSCLC. Therefore, p-JAK1 could be a target for early diagnosis and prognosis prediction.

In the present study, patients with p-JAK1$^+$ and EGFR duplication exhibited the poorest prognosis while patients with p-JAK1$^-$ and no EGFR duplication exhibited the most favorable prognosis (median survival time, 16 vs. 61.5 months). Although contradictory results were previously reported (14,15), EGFR FISH status alone was not associated with the patient survival times (data not shown). To date, few studies have focused on the interaction between EGFR and JAKs at the cellular and molecular level. However, it has been identified that crosstalk between the JAK/STAT and EGFR pathways mediates adenomatous polyposis coil 1-driven intestinal stem cell hyperplasia in drosophila (26). Furthermore, prolactin, a JAK2-coupled cytokine receptor, has also been demonstrated to synergistically augment EGF signaling in T47D breast cancer cells (27). In NSCLC, how EGFR and JAK1 interact to exert biological effects should be further elucidated.

The present study indicated that JAK1 activation is associated with a poor prognosis in NSCLC, particularly in lung ADCC. p-JAK1 is an independent poor prognostic factor and a potential target for early diagnosis. The combination of EGFR gene amplification and JAK1 activation may be a novel tool for prognosis prediction in patients with lung ADCC.

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