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Original Article

Smurf1-positive expression indicates favorable survival for resected non-small cell lung cancer patients

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Abstract: Recently studies have found that Smurf1 inhibits PIPKIγ-promoted lung cancer cell growth, tumorigenesis, and drug resistance through mediating the ubiquitination and degradation of PIPKIγi2. However, at present there is no study focused on the expression of Smurf1 protein as well as correlations among Smurf1 and lung cancer patients' survival. Therefore, we appraised Smurf1 expression by immmunohistochemistry and analyzed associations with prognosis in resected non-small cell lung cancer (NSCLC) patients. Overall, a number of 175 patients were enrolled in our study. We found that about 53 (30.2%) out of 175 NSCLC patients had Smurf1-pisitive expression. Smurf1-positive expression was significantly associated with lower lymph node metastasis (P=0.012). Smurf1-positive NSCLC patients had more favorable 5-year survival than patients with Smurf1-negative expression by univariate analysis (P=0.0002). Subgroup analysis found the same trend in lung adenocarcinoma (ADC, P=0.0006) other than lung squamous cell carcinoma (SCC, P=0.205). More interestingly, multivariate analysis also suggested that Smurf1-positive expression was significantly related to better overall survival (OS, P=0.003), independent of clinicopathological features and treatments of NSCLC patients. Unfortunately, we failed to observe statistically significant results when analyzed correlations among Smurf1 and NSCLC patients' progression-free survival (PFS, P=0.059). However, subgroup analysis revealed that Smurf1-positive patients had more favorable PFS for lung ADC patients (P=0.011) other than lung SCC (P=0.754). From the above, we guess that Smurf1 should be closely related to tumor metastasis and serve as an independent predictor of favorable prognosis in resected NSCLC patients.

Keywords: Smurf1, non-small cell lung cancer, survival

Introduction

Lung cancer is one of the most common malignancies and the leading cause of cancer-related deaths worldwide [1]. Of subtypes of lung cancer, Non-small cell lung cancer (NSCLC) accounts for about 85% in the United States, and until now only 18.7% of lung cancer patients can achieve 5-year survival [2]. Therefore, it is vital to find biomarkers to estimate lung cancer patients' prognosis and guide therapies.

Smad ubiquitination regulatory factor 1 (Smurf1), containing a WW domain, C2 domain and HECT domain, is a homolog to E6AP carboxyl Terminus (HECT) E3 ubiquitin ligase [3-5]. Recently, studies have found that Smurf1 inhibits PIPKIγ-promoted lung cancer cell growth, tumorigenesis, and drug resistance through mediating the ubiquitination and degradation of PIPKIγi2 [6]. For the moment, however, no study has appraised the expression and prognostic values of Smurf1 for NSCLC patients. Therefore, we performed the study herein to study the expression of Smurf1 protein in resected NSCLC tumors using immunohistochemistry (IHC) and appraise correlations among the Smurf1 protein expression and survival prognosis of NSCLC patients.

Materials and methods

Patients and specimens

Totally a number of 175 NSCLC patients with complete tumor resection in West China Hospital, Sichuan University were subsequently

Table 1. The base line data of enrolled patients in our study

Variables	Data (n=175)
Age (year, median, range)	56.8 (29-86)
Gender	
Male	120 (68.6%)
Female	55 (31.4%)
Smoking History	
Yes	107 (61.1%)
No	68 (38.9%)
Histological subtype	
ADC	92 (52.6%)
SCC	69 (39.4%)
Others	14 (8.0%)
Differentiation	
Poor	97 (55.4%)
Moderate-well	78 (44.6%)
T stage	
T1-2	131 (74.9%)
T3-4	44 (25.1%)
N stage	
N0	97 (55.4%)
N1-3	78 (44.6%)
M stage	
M0	166 (94.6%)
M1	9 (5.1%)
TNM stage	
I-II	110 (62.9%)
III-IV	65 (37.1%)
Radiotherapy	
Yes	37 (21.1%)
No	138 (78.9%)
Chemotherapy	
Yes	118 (67.4%)
No	57 (32.6%)
Smurf1	
Positive	53 (30.2%)
Negative	122 (69.7%)

ADC, Adenocarcinoma. SCC, Squamous cell carcinoma.

enrolled in our study from the year of 2008 to 2011. After surgery, all the patients received standard therapies according to the non-surgical management for lung cancer of Clinical Oncology Information Network guidelines. The exclusion criteria are as follows: Patients with previous malignancy, neoadjuvant therapy or incomplete clinical data. Tumor specimens were fixed in formalin and embedded in paraffin immediately after resection. Clinicopathological data including gender, age, smoking history, histological subtype, tumor differentiation, TNM stage and therapies were collected. Tumor staging was according to the tumor-node-metastasis (TNM) staging system of the America Joint Committee on Cancer (AJCC 7th edition) [7]. Histological subtypes were classified according to the World Health Organization classification for NSCLC [8].

Follow-ups for NSCLC patients continued until cancer-related deaths or more than five years after diagnosis. Overall survival (OS) was defined as the time interval from the date of primary diagnosis to the date of cancer-related deaths or the end of follow-up. Progression-free survival (PFS) was defined as the time interval from the date of surgery to the date of first documented disease progression or death [9]. Written informed consents were obtained from all patients and our study gained the approval of the Committee on Medical Ethics of West China Hospital, Sichuan University.

Immunohistochemistry

Paraffin-embedded tissues were made into 4 μm-thick sections. The primary antibody was mouse monoclonal IgG antibody purchased from Abcam Corporation (Product ID: ab57573). Secondary antibodies were goat anti-mouse antibodies purchased from Dako Corporation. We conducted the immunohistochemistry staining as described in our previous study [10]. The primary antibody was 1:200 diluted. Negative controls, primary antibodies replaced by phosphate-buffered saline (PBS), showed no immunoreactivity.

Evaluation the expression of Smurf1

Two pathologists blind to patient's clinical data conducted evaluations independently. Smurf1 protein was mostly membrane and cytoplasm positive in tumor samples. A total of three fields under light microscope were chosen for scoring according to the double semi-quantitative scoring as previously described [11]. Overall score ≥3 was defined as positive expression.

Statistical analysis

Statistical analysis were conducted using SPSS 19.0 (SPSS, Chicago, USA) and graphs were

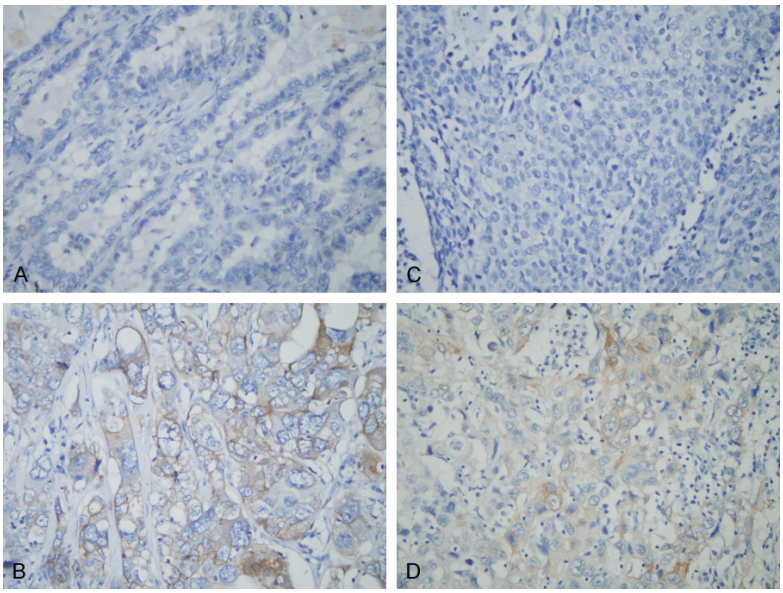

Figure 1. The expression of Smurf1 proteion in lung adenocarcinoma (A, negative; B, positive) and squamous cell carcinoma (C, negative; D, positive) using immunohistochemistry. Original magnification ×200.

made using Graphpad prism 6 (La Jolla, USA). Chi-square (χ^2) tests were employed to appraise the correlation among clinicopathological data and Smurf1 protein expression. The Kaplan-Meier curves and log-rank tests were used to evaluate the correlation among patients' survival and Smurf1 expression. A multivariate Cox regression analysis was performed to estimate independent prognostic factors. *P* values ≤0.05 were considered statistically significant.

Results

Clinicopathological features

As shown in **Table 1**, a total of 175 NSCLC patients were enrolled in our study. The patients' median age was 56.8 years old (range: 29-86 years old). Of the patients, a number of 120 (68.6%) cases were male, and 107 (61.1%) cases had smoking history. Patients diagnosed with adenocarcinoma accounted for 52.6%. Around 110 (62.9%) patients' TNM stage was I-II stage. About 37 (21.1%) cases had radiotherapy and 118 (67.4%) had chemotherapy after surgery.

Smurf1 expression in tumor samples

Smurf1 was positively stained mostly in membrane and cytoplasm in NSCLC tumor tissues. Smurf1 was positive expression in 53 (30.2%) samples, as shown in **Figure 1B** and **1D**, and the remaining 122 specimens (69.7%) was negative expression, as shown in **Figure 1A** and **1C**.

Correlations among Smurf1 expression and patients' clinicopathological features

The correlation among Smurf1 expression and clinicopathological features was demonstrated in **Table 2**. We found that Smurf1 was significantly associated with lymph node metastasis in NSCLC patients ($P<0.05$), results showing that 16 (30.2%) cases out of all Smurf1-positive patients had lymph node metastasis, significantly lower the ratio (50.8%) in Smurf1-negative patients ($P=0.012$), while no statistical significance was observed among Smurf1 expression and other clinicopathological variables (all $P>0.05$).

Correlations between Smurf1 expression and patients' survival

As shown in **Figure 2A**, about 80.0% of NSCLC patients with Smurf1-positive expression achieved five-year survival, significantly over more than 50.8% for Smurf1-negative NSCLC patients ($P=0.0002$). Subgroup analysis showed that no significant result was observed in lung squamous cell carcinoma (SCC) (**Figure 2B**) ($P=0.205$), but the five-year survival rate of the positive and negative groups was statistically significant in lung adenocarcinoma (ADC) (**Figure 2C**) ($P=0.0006$).

As shown in **Table 3** and **Figure 3A**, further analysis showed that the progression-free survival (PFS) rate for the Smurf1-positve and Smurf1-negative NSCLC patients was not statistically significant ($P=0.059$). Subgroup analysis showed that around 72.5% of Smurf1-positive lung ADC patients achieved 2-year PFS, significantly greater than the rate (35.5%) for Smurf1-negative lung ADC patients (**Figure 3C**) ($P=0.011$). No statistical significance for their

Table 2. Correlation among Smurf1 expression and patients' clinicopathological features

Variables	Smurf1 (n=175)		*P* value
	Positive (n=53)	Negative (n=122)	
Age (year)			0.197
≤65	45 (84.9%)	93 (76.2%)	
>65	8 (15.1%)	29 (23.8%)	
Gender			0.406
Male	34 (64.2%)	86 (70.5%)	
Female	19 (35.8%)	36 (29.5%)	
Smoking history			0.068
Yes	27 (50.9%)	80 (65.6%)	
No	26 (49.1%)	42 (34.4%)	
Histological subtype			0.745
ADC	29 (54.7%)	63 (51.6%)	
SCC	21 (39.6%)	48 (39.3%)	
Others	3 (5.7%)	11 (9.0%)	
Differentiation			0.649
Poor	28 (52.8%)	69 (56.6%)	
Moderate-well	25 (47.2%)	53 (43.4%)	
T stage			0.615
T1-2	41 (77.4%)	90 (73.8%)	
T3-4	12 (22.6%)	32 (26.2%)	
N stage			0.012*
N0	37 (69.8%)	60 (49.2%)	
N1-3	16 (30.2%)	62 (50.8%)	
M stage			0.248
M0	52 (98.0%)	114 (93.7%)	
M1	1 (2.0%)	8 (6.3%)	
Radiotherapy			0.470
Yes	13 (24.5%)	24 (19.7%)	
No	40 (75.5%)	98 (80.3%)	
Chemotherapy			0.190
Yes	32 (60.4%)	86 (70.5%)	
No	21 (39.6%)	36 (29.5%)	

*Statistically significant. ADC, Adenocarcinoma. SCC, Squamous cell carcinoma.

PFS between Smurf1-positve and Smurf1-negative patients was observed in lung SCC patients (**Figure 3B**) (*P*=0.754).

Finally we built multivariate Cox regression models taken into Smurf1 and clinicopathological variables and treatments, including age, gender, smoking history, T stage, N stage, M stage, tumor differentiation, radiotherapy and chemotherapy. Interestingly, we found that Smurf1-positive NSCLC patients had more favorable OS (HR=0.313, 95% CI: 0.145-0.676, *P*=0.003), shown in **Table 4**. Unfortunately, there was no statistical significance for PFS between the two groups (HR=1.499, 95% CI: 0.792-2.837, *P*=0.214), shown in **Table 4**.

Discussion

For the first time, we appraised the expression of Smurf1 protein in NSCLC tumors and studied the correlation among Smurf1 expression and NSCLC patients' clinicopathological features and survival. We found that Smurf1 was mostly membrane and cytoplasm staining in lung tumor samples and about 53 (30.2%) out of 175 NSCLC patients had Smurf1-pisitive expression. Smurf1-positive expression was significantly associated with lower lymph node metastasis in resected NSCLC patients (*P*=0.012). Smurf1-positive NSCLC patients had more favorable 5-year survival than patients with Smurf1-negative expression by univariate analysis (*P*= 0.0002). Subgroup analysis found the same trend in lung ADC (*P*=0.0006) other than lung SCC (*P*=0.205). More interestingly, multivariate analysis also suggested that Smurf1-positive expression was significantly related to better OS (*P*=0.003), independent of clinicopathological features and treatments of NSCLC patients. Unfortunately, we failed to observe statistically significant results when analyzed correlations among Smurf1 and NSCLC patients' PFS (*P*=0.059). However, subgroup analysis revealed that Smurf1-positive patients had more favorable PFS for lung ADC patients (*P*=0.011) other than lung SCC (*P*=0.754).

Smurf1, containing a WW domain, C2 domain and HECT domain, is a homolog to E6AP carboxyl Terminus (HECT) E3 ubiquitin ligase, which regulates several important signaling pathways, including the bone morphogenetic protein pathway and the transforming growth factor-beta (TGF-β) signaling pathway [3-5]. Overwhelming studies have found that Smurf1 is involved in cancer cell proliferation, cell plasticity, cell cycle, cell migration and invasion through binding to various proteins, including TRAF4, RhoA and Smad [12-14]. In lung cancer, studies have found that Smurf1 is involved in many biological processes of cancer cells.

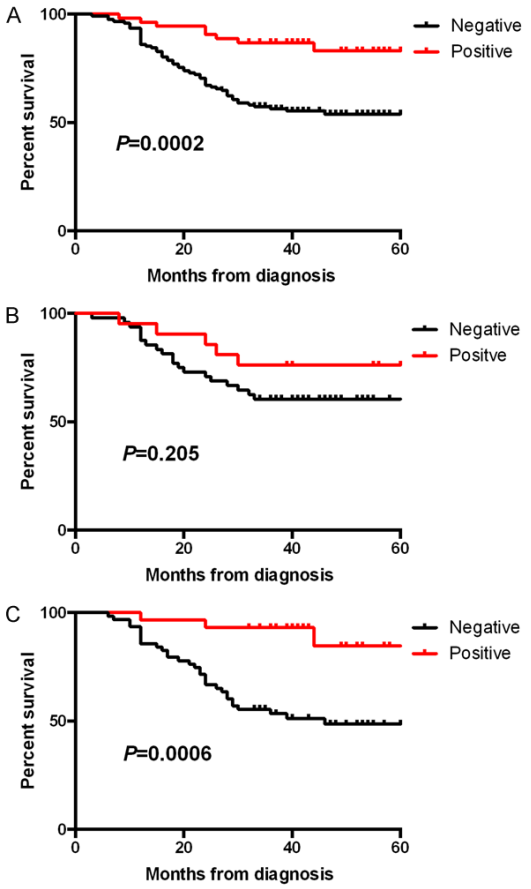

Figure 2. Kaplan-Meier survival curves showed the correlation between Smurf1 and overall survival in non-small cell lung cancers (NSCLC) (A), lung squamous-cell carcinomas (SCC) (B), and lung adenocarcinomas (SDC) (C).

Xie et al found Smurf1 ubiquitin ligase targets the transcriptional factor KLF2 for ubiquitination and degradation through the WW domain but not C2 or HECT domains binding to KLF2 in human lung cancer H1299 cells, consequently represses KLF2 activity and regulates the expression its downstream genes such as CD62L and Wee1 [15]. Previous studies have indicated that CD62L is crucial for KLF2 regulating T cell egress from the thymus and homing to the lymph nodes [16, 17]. The mechanism may explain the phenomenon found in our study that Smurf1-positive patients had lower lymph node metastasis. Besides, studies have found that PIPKIγ is highly expressed in lung cancer tissues and may be intimately correlated to their prognosis [6]. Overexpression of PIPKIγi2 can promote the lung cancer cell proliferation, tumorigenesis and drug resistance, and Smurf1 is crucial for the ubiquitination and degradation of PIPKIγi2 [6], which is in consistent with the finding in our study that Smurf1-positve NSCLC patients had more favorable survival. However, Wei et al have indicated that Smurf1 promotes S phase progression and stimulates proliferation of lung cancer cells largely by promoting degradation of the S phase suppressor protein Wee1 [18], which is opposite to our findings. Unfortunately, at present there are few studies focusing on the functions and mechanisms of Smurf1 in lung cancer cells, therefore it is difficult to explain the causes.

Fortunately, overwhelming evidence has found about Smurf1 in other tumors. Wang et al have found that the expression of Smurf1 is enhanced in hepatocellular carcinoma and transfection of Smurf1-specific siRNA can promote the apoptosis whilst inhibit the proliferation of hepatocellular cancer cells [19]. However, Tang et al have reported that Smurf1 act as tumor suppressors in liver cancer cells [20, 21]. Thus it can be seen that functions of Smurf1 in hepatocellular carcinomas remains controversy, exactly as the circumstances in lung cancer. Interestingly, Kwon et al have found that epidermal growth factor (EGF) increased Smurf1 expression at both the mRNA and protein levels in human breast cancer cell line and Smurf1 induction by EGF treatment or by the overexpression of MEK1 or Smurf1 resulted in enhanced cell migration and invasion, whereas SMURF1 knockdown suppressed EGF- or MEK1-induced cell migration and invasion. However, for the moment there is no research studied the correlation beween Smurf1 and EGFR in lung cancer. Thus it will be interesting and important in future that more studies focus on Smurf1.

In addition, our study showed that Smurf1 was not significantly related to OS of lung SCC patients. We guess the cause should be that there are only 69 cases of lung SCC patients, out of which a total of 21 cases positively express Smurf1 protein, therefore the sample size is too small to explain the issue. Thus, more studies with Large-scale samples are needed to further study the issue.

From the above, we guess that Smurf1 should be closely related to tumor metastasis and serve as an independent predictor of favorable prognosis in resected NSCLC patients, but

Table 3. Corrrelations among Smurf1 and patient' survival by univariate analysis

Variables	OS			PFS		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Smurf1 in NSCLC (positive/negative)	0.274	0.221-0.624	<0.001*	0.573	0.264-1.008	0.059
Smurf1 in SCC (positive/negative)	0.536	0.245-1.356	0.205	0.876	0.358-2.089	0.754
Smurf1 in ADC (positive/negative)	0.167	0.145-0.586	<0.001*	0.350	0.222-0.804	0.011*

*Statistically significant. OS, overall survival. PFS, progression-free survival.

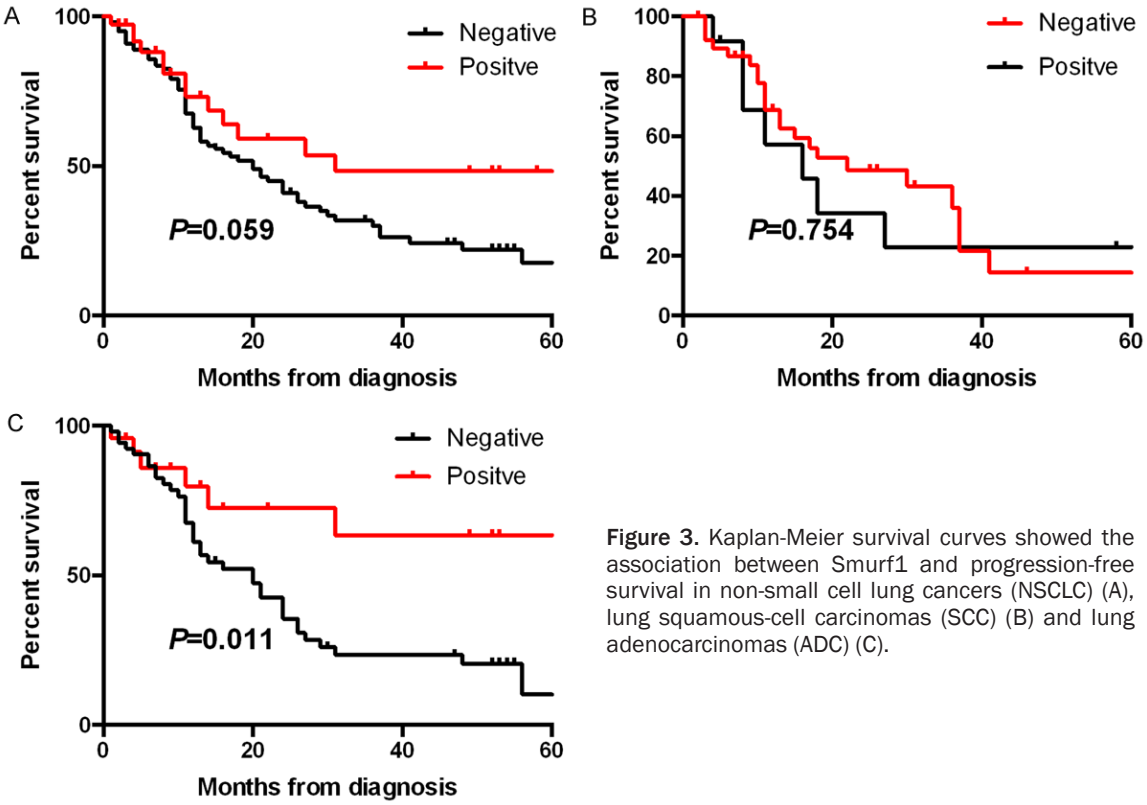

Figure 3. Kaplan-Meier survival curves showed the association between Smurf1 and progression-free survival in non-small cell lung cancers (NSCLC) (A), lung squamous-cell carcinomas (SCC) (B) and lung adenocarcinomas (ADC) (C).

Table 4. Corrrelations among Smurf1 and patient' survival by Multi-variate analysis

Variables	OS			PFS		
	OR	95% CI	*P* value	OR	95% CI	*P* value
Smurf1 in NSCLC (positive/negative)	0.313	0.145-0.676	0.003*	1.499	0.792-2.837	0.214

*statically significant. OS, overall survival. PFS, progression-free survival.

more researches are needed in future to confirm correlations between Smurf1 and lung SCC patients' survival and further study functions and mechanisms of Smurf1 in lung cancer.

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Disclosure of conflict of interest

None.

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