SRPX2 promotes cell proliferation and invasion via activating FAK/SRC/ERK pathway in non-small cell lung cancer

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INTRODUCTION

Lung cancer has one of the world’s highest incidence and mortality among the malignant tumors, and is the leading cause of cancer death and poses a serious threat to human life and health (Jemal et al., 2010). Lung cancer can be divided into two categories according to its pathological features: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC, including adenocarcinoma, large-cell carcinoma, squamous cell carcinoma and adenosquamous carcinoma) (Youdel & Baade, 2008). In fact, NSCLC is the major type of lung cancer accounting for about 80% to 85% of the total lung cancer cases (Gridelli et al., 2015). However, due to the lack of molecular markers for early diagnosis, more than half of NSCLC patients suffer from late clinical diagnosis, leading to cancer cell invasion and metastasis. The five-year overall survival (OS) rate is only about 17% (Gu et al., 2016). Therefore, studying the pathogenesis of NSCLC and finding efficient early diagnostic markers are critical for the discovery of therapeutic targets and improving OS rate.

The local microenvironment of cancer cells plays an indispensable role in the progression of cancer. The main component of the microenvironment is the extracellular matrix (ECM), which mainly includes glycoproteins, proteoglycans and collagen (Caterson, 2012). These molecules are important for tissue and organ morphogenesis and for the maintenance of cell and tissue structure and function (Özbek et al., 2010; Rosso et al., 2004). The binding of ECM to cell surface receptors (such as integrins) can initiate a series of signaling cascades that control cell survival, differentiation, proliferation, invasion, migration, apoptosis etc. (Jin & Varner, 2004; Rozario & DeSimone, 2010; Wickström & Fässler, 2011). Thus, it seems that ECM dysfunction is one of the important features in tumor progressions, and abnormal ECM can lead to the infiltration and metastasis of tumor cells (Venning & Erler, 2015; Naba et al., 2013; Malandrino et al., 2018; Pickup & Weaver, 2014; Lemberg, 2010). Sushi repeat containing protein X linked 2 (SRPX2) was firstly discovered in leukemia cells. It is a chondroitin sulfate proteoglycan (Kurosawa et al., 1999). Increasing evidence suggested that mutations of SRPX2 cause seizures (Royé-Zemmour et al., 2008; Salmi et al., 2013), linguistic and cognitive dysfunction (Roll et al., 2006), and mental retardation (Sia & Huganir, 2013). Recent studies showed that SRPX2 protein can also participate in the development of a variety of malignant tumors including gastric cancer (Tanaka et al., 2009; Tanaka et al., 2012), colorectal cancer (Öster et al., 2013; Liu et al., 2015), pancreatic...
cancer (Gao et al., 2015) and glioblastoma (Tang et al., 2016). The accumulating evidence that SRPX2 is important for tumorigenesis and development suggests that SRPX2 may be a valuable new target for tumor treatment. Nevertheless, its function and molecular mechanism of action in NSCLC have not yet been elucidated.

In this study, we analyzed the expression of SRPX2 in NSCLC tissues and explored the correlation between SRPX2 expression and clinicopathological results. In addition, the role of SRPX2 in NSCLC cell proliferation, migration and invasion was analyzed both in the in vitro and in vivo experiments. Moreover, the potential regulatory mechanism in NSCLC was also proposed.

MATERIALS AND METHODS

Patients and Tissues. This study was performed in accordance to the tenets of the Declaration of Helsinki and the ethical guidelines for medical and health research of the National Institutes of Health (NIH) and approved by the Ethics Committees of Wuhan NO.1 Hospital (Wuhan, Hubei Province, China). A total of 46 pairs of NSCLC tissue samples and matched adjacent normal tissue samples were obtained from patients who had surgery at Wuhan NO.1 Hospital. All enrolled patients gave their written informed consent.

Immunohistochemistry (IHC) assay. NSCLC and adjacent normal tissue samples of the enrolled patients and mouse tumor tissues were fixed, embedded, sliced into 4-mm sections and incubated with primary antibodies: anti-SRPX2, anti-Ki-67, and anti-E-cadherin (Abcam, Cambridge, MA, USA) at 4°C overnight. Then, the sections were incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. The peroxidase was detected using 3,3′-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin.

Cell culture. The normal human bronchial epithelial cell line (16HBE) and human NSCLC cell lines (A549, H1975, SPC-A1, H1229 and H460) were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). All the cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Inc., Waltham, MA, USA) pre-coated with 100 μg Matrigel (BD Biosciences, Bedford, MA, USA). 500 μl of serum-free medium and 400 μl of DMEM medium with 10% FBS were separately added into the upper and lower chamber, medium and 400 μl of DMEM medium with 10% FBS was cultured in 6-well plates to reach 85% confluence, and cultured in 6-well plates to reach 85% confluence, and were fixed in paraformaldehyde (PFA, Sigma-Aldrich, St. Louis, MO, USA) and stained with 0.5% crystal violet (Beijing Solarbio Science & Technology Co.Ltd, Beijing, China). Stained cells were washed in PBS, then photographed using a microscope and counted (Olympus Corporation).

Western blot. Cells (1×10⁴ cells/well) transfected with indicated plasmids were seeded onto a 6-well culture plate and incubated for 24 hours. Proteins were extracted from tissues and cells using RIPA lysis buffer (Beyotime Institute of Biotechnology), and were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% nonfat milk for 1 h, the membranes were incubated with primary antibodies as follows: anti-SRPX2 (1:1000, ab91584, Abcam), anti-PCNA (1:1000, ab29532, Abcam), anti-E-cadherin (1:500, ab15148, Abcam), anti-N-cadherin (1:1000, ab76057, Abcam), anti-FAK (1:2000, ab40794, Abcam), anti-phospho-FAK (Tyr576/577, 1:1000, #3281, Cell Signaling Technology, Beverly, MA, USA), anti-Erk1/2 (1:1000, #9102, Cell Signaling Technology), anti-phospho-Erk1/2 (Tyr202/Tyr204, 1:1000, #4377, Cell Signaling Technology), anti-SRC (1:1000, #2108, Cell Signaling Technology), anti-phospho-SRC (Tyr416, 1:1000, #2101, Cell Signaling Technology) and anti-GAPDH (1:10000, ab181602, Abcam). Subsequently, membranes were probed with corresponding secondary antibody (Abcam), visualized by ECL reagent (Invitrogen) and detected using an imaging system (Bio-Rad, Herzeu, CA, USA).

Cell counting kit-8 (CCK-8) assay. Briefly, 1000 cells/well were seeded onto 96-well plates and transfected. 10 μL of CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) was used according to the manufacturer's instructions: added to each well and incubated for 2 hours. The absorbance was measured at 450 nm using a Microplate Autoreader (Thermo Fisher Scientific, Inc.) 24, 48, 72 and 96 hours after transfection.

Colony formation assay. 1000 cells/well were maintained in 6-well plates with DMEM medium replaced every 3 days for two weeks. The colonies were subsequently fixed in paraformaldehyde (PFA, Sigma-Aldrich, St. Louis, MO, USA) and stained in 0.5% crystal violet (Beijing Solarbio Science & Technology Co.Ltd, Beijing, China). Stained cells were washed in PBS, then photographed using a microscope and counted (Olympus Corporation, Tokyo, Japan).

Wound healing assay. Cells (1×10⁴ cells/well) were cultured in 6-well plates to reach 85% confluence, and then mitomycin C (10 μg/ml, Sigma-Aldrich) was added to the cells for 2 hours. A wound was scratched in the cell monolayer in each plate with a 200 μl pipette tip. The cell debris was removed by PBS wash and the remaining cells were maintained in serum-free medium. At 0 h and 24 h after the scratch, the images were recorded using a microscope (Olympus Corporation).

Transwell assay. 1×10⁴ cells/well were seeded onto the upper chamber (Corning Life Sciences, Tewksbury, MA, USA) pre-coated with 100 μg Matrigel (BD Biosciences, Bedford, MA, USA). 500 μl of serum-free medium and 400 μl of DMEM medium with 10% FBS were separately added into the upper and lower chamber, respectively. After 24 hours, cells in the lower chamber were fixed in 4% PFA, stained with 0.5% crystal violet and counted under a microscope (Olympus Corporation).

Tumor xenograft. The animal experiments were performed according to the guidelines for the care and use of laboratory animals with the approval of Wuhan NO.1 Hospital and the Guide for NIH and the institutional...
SRPX2 promotes cell proliferation and invasion

Ethical guidelines for animal experiments. 5-week-old female BALB/c nude mice were obtained from Shanghai Experimental Animals Centre (Shanghai, China) and randomly divided into two groups: sh-NC and sh-SRPX2. A549 cells (5×10^6) transfected with sh-NC or sh-SRPX2 were injected subcutaneously into either flank of the mouse (n=5 per group). The tumor width and length were measured every 3 days and the tumor volume was calculated using the formula: tumor volume (mm³) = length (mm)×width (mm)²×0.5. After 21 days, mice were euthanized and tumors were excised and weighed. The tumor tissues were fixed for subsequent IHC assay.

**Statistical analysis.** Data were analyzed using a Statistical product (SPSS, version 20.0, SPSS Inc., Chicago, Illinois, USA) and presented as Mean ± S.D. from three independent experiments. Overall survival curves were analyzed by Kaplan–Meier method and log-rank test. Comparison between two or more groups was performed by student’s t-test or one-way ANOVA, respectively. P<0.05 was regarded to indicate a statistically significant difference.

**RESULTS**

SRPX2 is upregulated in NSCLC tissues and associated with poor prognosis

To confirm the dysregulation of SRPX2 in NSCLC, the mRNA level of SRPX2 was analyzed by qRT-PCR. Results (Fig. 1A) indicated that SRPX2 was considerably upregulated in NSCLC tissues (Tumor) compared to the adjacent non-tumor tissues (Non-tumor). Similarly, western blot and IHC assays also confirmed the upregulation of SRPX2 in NSCLC tissues compared to that in non-tumor tissues (Fig. 1B–C). Further statistical analysis on the relationship between SRPX2 expression and clinical pathological data in NSCLC patients was summarized in Table 1. The results revealed that the upregulation of SRPX2 was related to tumor size (P=0.021), lymph node metastasis (P=0.015), distant metastasis (P=0.038) and clinical stage (P=0.027) in NSCLC patients (n=46). The correlation between SRPX2 expression and prognosis of NSCLC patients was analyzed via Kaplan–Meier survival analysis...
analysis. As depicted in Fig. 1D, the high expression of SRPX2 correlated with significantly lower overall survival (OS) than the low expression of SRPX2 (*P*=0.0086). These results revealed that high SRPX2 expression predicted poor prognosis of patients with NSCLC. SRPX2 was also upregulated in NSCLC cell lines (A549, H1975, SPC-A1, H1229 and H460) as compared to human bronchial epithelioid cell line 16HBE (Fig. 1E–F). A549 cells with higher expression of SRPX2 were selected for the subsequent loss-of-function assays, while SPC-A1 cells with lower expression of SRPX2 were used for gain-of-function assays.

**SRPX2 promotes proliferation in NSCLC cells**

The transfection efficiency of SRPX2 in SPC-A1 and A549 cells was validated by western blot assay. As illustrated in Fig. 2A, SRPX2 group displayed a significant increase in SRPX2 levels, indicating the successful transfection. Sh-SRPX2#1 group with a higher SRPX2 knockdown efficiency was chosen for the subsequent loss-of-function assays and named as sh-SRPX2. CCK-8 and colony formation assays demonstrated that upregulation of SRPX2 dramatically promoted the proliferation of SPC-A1 cells, whereas knockdown of SRPX2 significantly decreased the proliferation of A549 cells (Fig. 2B–C). Taken together, these data uncovered that SRPX2 might contribute to the progression of NSCLC by increasing cell proliferation.

**SRPX2 promotes migration and invasion of NSCLC cells**

As presented in Fig. 3A, wound healing assay revealed that the migratory capacity of SPC-A1 cells was evidently increased by SRPX2 overexpression, whereas the migration of A549 cells was reduced by SRPX2 knockdown. The transwell assay showed a significantly increased number of invasive cells when SRPX2 was overexpressed, whereas SRPX2 knockdown led to significantly decreased number of invasive cells (Fig. 3B). These data indicated that SRPX2 promoted NSCLC cell migration and invasion.

### Table 1. Correlation between expression of SRPX2 and clinicopathological Characteristics in NSCLC patients (n=46).

| Variables                          | n   | SRPX2          | P value |
|-----------------------------------|-----|----------------|---------|
|                                   |     | High expression (n=25) | Low expression (n=21) |
| Gender                            |     |                |         |
| Male                              | 16  | 7              |         |
| Female                            | 30  | 16             | 14      | 0.850 |
| Age(years)                        |     |                |         |
| ≥65                               | 18  | 9              |         |
| <65                               | 28  | 16             | 12      | 0.635 |
| Smoking                           |     |                |         |
| Yes                               | 19  | 11             | 8       |
| No                                | 27  | 13             |         |
| Pathology                         |     |                |         |
| Squamous cell carcinoma           | 18  | 10             | 8       |
| Adenocarcinoma                    | 26  | 12             |         |
| Others                            | 2   | 1              | 1       | 1.000 |
| Differentiation                    |     |                |         |
| Well differentiated               | 11  | 6              |         |
| Moderately differentiated         | 18  | 9              |         |
| Lowly or undifferentiated         | 17  | 11             | 6       | 0.543 |
| T classification                   |     |                |         |
| T1+T2                            | 29  | 17             |         |
| T3+T4                            | 17  | 13             | 4       | 0.021 |
| N classification                   |     |                |         |
| N0+N1                             | 31  | 18             |         |
| N2+N3                             | 15  | 12             | 3       | 0.015 |
| Distant metastasis                |     |                |         |
| M1                                | 5   | 5              |         |
| M0                                | 41  | 20             | 21      | 0.038 |
| Clinical stage                     |     |                |         |
| I+II                              | 27  | 11             | 16      |         |
| III+IV                            | 19  | 14             | 5       | 0.027 |

Bold values signifies *P*<0.05
SRPX2 promotes cell proliferation and invasion in NSCLC cells

Further analysis compared the levels of cell growth- and EMT-related proteins in SPC-A1 and A549 cells. Overexpression of SRPX2 increased the protein levels of PCNA and N-cadherin and decreased the protein level of E-cadherin (Fig. 4A). Meanwhile, knockdown of SRPX2 caused the opposite results (Fig. 4B). As presented in Fig. 4A and 4B, overexpression of SRPX2 dramatically increased the phosphorylation levels of FAK (p-FAK), SRC (p-SRC) and ERK1/2 (p-ERK1/2), while the loss of SRPX2 decreased the phosphorylation levels...
of these proteins. Therefore, these data indicated that SRPX2 promoted EMT and activated FAK/SRC/ERK signaling in NSCLC.

**SRPX2 promotes tumor growth in xenograft mice**

A549 cells with stable knockdown of SRPX2 were inoculated into nude mice. 21 days later, the subcutaneous tumors were harvested, and SH-SRPX2 group had smaller tumors than SH-NC group (Fig. 5A). The volume and weight of tumors were also obviously decreased in SH-SRPX2 group as compared with the SH-NC group (Fig. 5B–C). IHC staining (Figure 5D) indicated that the expression of SRPX2, Ki-67 and E-cadherin in tumors was determined by IHC. All data were presented as the mean ± S.D.; data represent three independent experiments. ***P<0.001.

**DISCUSSION**

This is the first evidence confirming that SRPX2 is up-regulated in NSCLC tissues and cells, and high SRPX2 expression predicts poor prognosis and is associated with a range of clinicopathological characteristics, including tumor size, lymph node metastasis, distant metastasis and clinical stage. Moreover, we found that SRPX2 could promote NSCLC cell proliferation, migration and invasion in vitro, and promoted tumor growth in vivo. Interestingly, further experiments displayed that SRPX2 regulated the development of NSCLC via activating FAK/SRC/ERK pathway.

Lung cancer is surrounded by extensive ECM in both primary and metastatic sites, and is associated with poor prognosis (LIM et al., 2017). ECM provides spatial support for cell-to-cell and cell-to-matrix interac-
tions (Lochter & Bissell, 1995; Radisky & Bissell, 2002). Numerous studies showed that ECM and its receptors are involved in the continuous progression of malignant transformation and metastasis (Ou et al., 2014; Liu, Weaver 7 Werb, 2012; Netti et al., 2000). The poor prognosis of NSCLC patients results from tumor metastasis and recurrence, which are closely related to the increased cancer cell mobility (Bremnes et al., 2002; He et al., 2018). Therefore, exploring the molecular mechanism is helpful for the therapy and diagnosis of NSCLC.

SRPX2, having the molecular mass of 53 kD, is located on chromosome Xq22.1 and is a downstream target gene of the E2A-HLF fusion gene (Sia et al., 2013; Kurosawa et al., 1999). Wilson identified SRPX2 as a chondroitin sulfate proteoglycan with the feature of ECM protein (Tanaka et al., 2012; Wilson et al., 2012). SRPX2 is abnormally expressed in a variety of tumors and could be used as a marker for tumor diagnosis. Lin et al. found that SRPX2 was highly expressed in and acted as an independent prognostic predictor of hepatocellular carcinoma (Lin et al., 2017). In addition, SRPX2 was also identified as a prognostic biomarker for prostate cancer (Zhang et al., 2018), pancreatic cancer (Li et al., 2019), gastric cancer (Tanaka et al., 2009), and glioblastoma (Tang et al., 2016).

More specifically, several previous studies indicated that SRPX2 exhibits carcinogenic function and was considered to be associated with malignant progression via different regulatory mechanisms. Gao and others (Gao et al., 2015), demonstrated that silencing of endogenous SRPX2 expression reduces migration and invasion of pancreatic carcinoma cells by regulating FAK-dependent signaling SRPX2 was also proved to promote the migration and invasion of hepatoma carcinoma cells by regulating the FAK/ AKT signaling (Lin et al., 2017). Moreover, SRPX2 intensified the EMT process and promotes glioma metastasis through the MAPK signaling pathway (Tang et al., 2016). A recent study clarified the new mechanism of SRPX2 promoting the proliferation and metastasis and inhibiting the chemical sensitivity through inactivation of the Wnt/β-catenin pathway in esophageal squamous cells (He et al., 2019). Nevertheless, no studies have considered the biological effect of SRPX2 on the development and progression of NSCLC. Our study indicated that SRPX2 could promote NSCLC cell proliferation, migration and invasion in vitro. In agreement with the in vitro results, knockdown of SRPX2 inhibited tumor growth and promoted the expression of epithelial marker (E-cadherin).

Focal adhesion kinase (FAK) and steroid receptor coactivator (c-Src) can regulate cytoskeleton dynamics and cell movement by affecting actin polymerization and focal adhesion inversion (Fife & Kavallaris, 2014). Phosphorylated FAK and SRC form complexes that activate or inhibit multiple downstream signaling, including P13K/ AKT, P53, ERK etc. The FAK/SRC/ERK signaling pathway plays important roles in the tumorigenesis and metastasis of various cancers, including NSCLC (Roy-Lazzarraga & Hodivala-Dilke, 2016; Patel et al., 2016; Kohno & Pouyssegur, 2006). For instance, PIG3 enhances cell migration and invasion via promoting the Fak/Src pathway in lung adenocarcinoma (Gu et al., 2018). Maclurin inhibits migration and invasion of NSCLC cells through repressing the Src/FAK–ERK–β-catenin signaling (Ke et al., 2015). Our study identified SRPX2 as a regulator of FAK/SRC/ERK signaling, evidenced by the increased levels of p-FAK, p-SRC p-ERK, which is consistent with previous studies (Gao et al., 2015; Lin et al., 2017). Besides, SRPX2 enhanced the protein levels of PCNA and N-cadherin, inhibited E-cadherin protein expression. These data confirmed that SRPX2 activated FAK/SRC/ERK signaling, regulated cell growth, modulated EMT-related proteins, and finally promoted NSCLC progression.

To sum up, this study uncovered the biological significance of SRPX2 in the development and progression of NSCLC. SRPX2 overexpression increased NSCLC cell proliferation, migration and invasion, which partly relied on the activation of FAK/SRC/ERK signaling. These results indicated that proteins of SRPX2-FAK/SRC/ERK axis may be potential therapeutic targets and prognostic markers for NSCLC.

Conflicts of interest

There was no conflict of interest.

Availability of data and materials

All data generated or analyzed in this study are included in this article.

Authors’ contributions

Xiujuan Li, Jing Liu and Hong Sun conceived and designed the experiments, Yong Zou, Juan Chen and Yi-chun Chen analyzed and interpreted the results of the experiments, Cheng Chen and Xuan Wu performed the experiments.

Ethics approval and consent to participate

The animal use protocol had been reviewed and approved by the Ethics Committees of Wuhan NO.1 Hospital.

Patient consent for publication

Not Applicable.

REFERENCES

Bremnes RM, Veve R, Hirsch FR, Franklin WA (2002) The E-cadherin cell–cell adhesion complex and lung cancer invasion metastasis and prognosis. Lung Cancer 36: 115–124. https://doi.org/10.1016/S0169-5002(01)00471-8

Caterson B (2012) Fell-Muir Lecture: Chondroitin sulphate glycosaminoglycans: fun for some and confusion for others. Int J Exp Pathol 93: 1–10. https://doi.org/10.1111/j.1365-2613.2011.00807.x

Fife C, McCarroll J, Kavallaris M (2014) Movers and shakers: cell cytoskeleton in cancer metastasis. Br J Pharmacol 171: 5507–5523. https://doi.org/10.1111/bph.12704

Gao Z, Zhang J, Bi M, Han X, Han Z, Wang H, Ou Y (2015) SRPX2 promotes cell migration and invasion via FAK dependent pathway in pancreatic cancer. Int J Clin Exp Pathol 8: 4791–4799

Gridelli C, Rossi A, Carbone DP, Guarize J, Karachaliou N, Mok T, Petrella F, Spaggiari L, Rosell R (2015) Non-small-cell lung cancer. Nat Rev Dis Primers 1: 13009. https://doi.org/10.1038/nrdp.2015.9

Gu MM, Gao D, Yao PA, Yu I, Yang XD, XiongG, Zhou J, Zhang ZF, Li M (2018) p53-inducible gene 3 promotes cell migration and invasion by activating the FAK/Src pathway in lung adenocarcinoma. Cancer Sci 109: 3783. https://doi.org/10.1111/cas.13818

He F, Wang H, Li Y, Liu W, Gao X, Chen D, Wang Q, Shi G (2019) Prognostic value of platelet to lymphocyte ratio in non-small cell lung cancer: evidence from 3430 patients. Sci Rep 9: 23893. https://doi.org/10.1038/s41598-019-43176-6

He W, Zhang H, Wang Y, Zhou Y, Luo Y, Cui N, Jiang W, Jiang H, Wang H, Xu D (2018) CTHRC1 induces non-small cell lung cancer (NSCLC) invasion through upregulating MMP-7/MMP-9. BMC Cancer 18: 400. https://doi.org/10.1186/s12885-018-4317-6

Vol. 67

SRPX2 promotes cell proliferation and invasion 171
