

Sideroflexin 1 Detection as a Predictive and Prognostic Biomarker in Non-small Cell Lung Cancer and Its Correlation With Clinical and Pathological Parameters

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Research article

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Abstract

Background

Non-small cell lung cancer (NSCLC) remained the leading cause of cancer-associated deaths worldwide. Increasing evidence showed that sideroflexin 1 (SFXN1) may function as an essential and novel regulator in various diseases. However, its mechanism and pathological role in NSCLC remained unclear.

Methods

In this study, we recruited 237 NSCLC patients. The demographic and pathological information of NSCLC patients were recorded and analyzed. The expressions of SFXN1 in tissues and cell lines were confirmed by qRT-PCR assay. Furthermore, the diagnostic and prognostic potentials of SFXN1 in NSCLC were detected by ROC and Kaplan-Meier analysis, respectively. CCK-8, wound healing and Transwell assays determined the cell viability, apoptosis, migration and invasion of A549 cell after transfection. Finally, PCNA, Bcl-2, Bax, MMP2, and MMP9 levels after knocking down SFXN1 expression in A549 cells were detected by western blot assay.

Results

We found that SFXN1 was significantly increased in NSCLC tumor tissues and cell lines. Up-regulated SFXN1 was associated with tumor size, lymph node metastasis, and TNM stage. The ROC analysis confirmed the high sensitivity, specificity, and accuracy of SFXN1 in discriminating tumor tissues from non-tumor adjacent tissues. Furthermore, Kaplan-Meier analysis revealed that increased expression of SFXN1 might predict unfavorable prognosis and disease-free survival rate. Knockdown of SFXN1 led to increased cell apoptosis and decreased cell proliferation, migration, and invasion in A549 cells. SFXN1 silencing generated decreased PCNA, Bcl-2, MMP2, MMP9 expressions, and increased Bax expression in A549 cells.

Conclusion

The results in our study suggested that SFXN1 was up-regulated in NSCLC, functioning as a predictive indicator for NSCLC diagnosis and prognosis, shedding new sights for therapeutic target treatment.

Background

Lung cancer was one of the most lethal malignancies worldwide, generating about 1.8 million new cancer cases and causing about 1.6 million deaths each year [1]. Lung cancer was a heterogeneous group of more than 50 histomorphological subtypes [2], usually classified as small cell lung cancer and non-small cell lung cancer (NSCLC). Among them, NSCLC accounted for about 75%-80%, which can be further divided into large cell lung cancer, lung adenocarcinoma, and squamous cell carcinoma [3]. Despite the success of targeted and immune-based therapies, their clinical outcomes were still lacking. This was mainly due to the lack of apparent disease symptoms and patients were often diagnosed at an advanced

stage, who cannot undergo surgery to remove all of the cancerous tissues. Patients with progressive diseases were mostly metastatic patients, and their 5-year survival rate is only 4.5%, which seriously affects their prognosis [4, 5]. In view of this, there is an urgent need to identify reliable diagnostic biomarkers for NSCLC to provide early intervention and reduce mortality.

Computed tomography (CT) was still the mainstay of lung-related diseases screening [6] CT was an effective method for diagnosing and monitoring lung tissue or airway diseases with high accuracy, allowing imaging of the disease site to determine its progression and guiding treatment [6]. However, routine CT screening methods were not widely available since it was relatively expensive and complicated to perform, significantly limiting their clinical application [7]. Cytotoxic chemotherapy remained an important modality for the treatment of metastatic NSCLC [8]. However, the prognosis after treatment remained unsatisfactory. This may be due to the lack of practical prognostic markers that can monitor the progression and metastasis of NSCLC which may help clinicians develop more appropriate treatment strategies for patients. Therefore, finding a candidate that can serve as a predictor of NSCLC treatment and prognosis was of great importance for treatment.

In this study, we aimed to investigate the relationship between SFXN1 and n NSCLC progression Our findings revealed that SFXN1 expression was higher in tumor tissues and cells than in paraneoplastic tissues and human normal lung epithelial cell. Its expression also correlated with TNM stage, lymph node metastasis, and tumor size. Functional studies showed that knockdown of SFXN1 reduced the proliferation, migration, and invasion capacities of NSCLC cells and enhanced apoptosis. Thus, our results show that silencing SFXN1 inhibited NSCLC progression and served as a potential target gene, providing new NSCLC treatment strategies.

Methods

Patients and samples

NSCLC tumor tissues and adjacent non-tumor tissues (3 cm away from the tumor's edge) were collected from 237 patients from the Affiliated Huaian No.1 People's Hospital of Nanjing Medical University between April 2013 and December 2014. The demographic and pathological characteristics were summarized in Table 1. All patients did not receive any chemotherapy or adjuvant therapy before the surgical section. The inclusion criteria were as follows: all patients were diagnosed with NSCLC by CT and pathologically diagnosed during surgery; all patients with other types of cancers other than NSCLC were excluded. This study obeyed the Declaration of Helsinki and written informed consent was provided by each patient. The Ethics Committee of the Affiliated Huaian No.1 People's Hospital of Nanjing Medical University approved this study.

Table 1

Relationship of aberrant SFXN1 expression with clinicopathological features in all NSCLC patients

Clinicopathological features		N	SFXN1 expression		P value
			High (n = 139)	Low (n = 98)	
Gender	Female	104	56	48	0.1842
	Male	133	83	50	
Age (years)	≤ 60	96	57	39	0.8516
	> 60	141	82	59	
TNM stage	I ~ II	128	58	70	< 0.0001
	III ~ IV	109	81	28	
Lymph node metastasis	Yes	120	86	34	< 0.0001
	No	117	53	64	
Smoking	Yes	153	93	60	0.3878
	No	84	46	38	
Tumor size (cm)	≤ 3	142	69	73	0.0001
	> 3	95	70	25	
Differentiation	Low	138	78	60	0.4322
	Moderate/High	99	61	38	

Cell culture

Human NSCLC lines A549, HCC827, H1299, NCI-H460, and human normal lung epithelial cells BEAS-2B were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences and used for this experiment. RPMI-1640 medium containing FBS and penicillin-streptomycin was used as a nutrient source for the cells, and cells were continuously cultured at 37°C with 5% CO₂ in a thermostatic cell incubator.

Cell transfection

In this experiment, si-SFXN1#, si-SFXN1* and their negative control si-NC, were synthesized by GenePharma and transfected into A549 cells using lipofectamine 2000 reagent to interfere with the expression of SFXN1. Briefly, A549 cells were inoculated into 96-well plates at a density of 5×10^3 cells/well and then incubated in an incubator containing 5% CO₂ at 37°C for 24 hours. When cell growth

reached 70%-80% confluence, cell transfection experiments were started under the manufacturer's instructions.

qRT-PCR assay

To detect whether there were differential expressions of SFXN1 in normal and lung cancer tissue or cells and whether si-SFXN1#, si-SFXN1*, and si-NC were successfully transfected into A549 cells, the relative expressions of SFXN1 at the RNA level in each corresponding group was detected by qRT-PCR, respectively. In brief, TRIzol reagent was used to lyse and extract total RNA from cells or tissues. Then, a NanoDrop microspectrophotometer was applied to quantify the RNA concentrations extracted from samples. cDNA was synthesized from RNA using Multiscribe RT kit, and q RT-PCR was then conducted by Universal SYBR Green Master Kit on an ABI PRISM 7500 PCR machine (Applied Biosystems). The PCR thermal cycles were as follows: 95°C for 1 minutes, 40 cycles of 95°C for 15 seconds, and 62°C for 45 seconds. Finally, solution curves were drawn, and the expressions of SFXN1 were quantified by equation $2^{-\Delta\Delta CT}$ method.

Western blot assay

Total proteins were extracted from tissues or cells using RIPA buffer and quantified by the BCA method following the manufacturer's instructions. Then, 20 µg/lane protein was separated by 10% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% skimmed-milk at 37°C for 50 minutes, membranes were incubated with primary antibodies at 4°C overnight. Following the primary incubation, the membranes were washed with PBS/Tween-20 (0.03%) and further incubated with secondary antibody at 37°C for 2 hours. Finally, protein bands were visualized with ECK and analyzed with ImageJ version 1.46 software (National Institutes of Health).

Cell viability measured by CCK-8 assay

To determine whether the cell viability of A549 cells was affected after interfering with si-SFXN1, the CCK-8 assay was measured accordingly. After transfection with si-SFXN1 or si-NC, cells were inoculated into 96-well plates, and the cell concentration was adjusted to the density of 5×10^3 cells/well. Then, cells were placed in a humidified incubator containing 5% CO₂ at 37°C for 24 hours. Afterward, 10 µL CCK-8 solution was added into each well with a pipette gun and incubated for another 1 hour at room temperature. After the incubation, the absorbance value was measured at 450 nm using a fully automatic quantitative mapping enzyme scale. The cell viability of each group was calculated. Three parallel copies were set for each experimental group, and the experiment was repeated three times.

Cell apoptosis analysis

At 24 hours post-transfection, cells were inoculated in a six-well density of 1×10^5 cells/well and incubated for 24 hours at 37°C in a humidified environment containing 5% CO₂. The cells were then collected for apoptosis detection. According to the Annexin V-FITC staining kit's instructions, cells were stained with

ethylenediaminetetraacetic acid (EDTA)-free and digested with 0.25% trypsin. After washing the cells twice with PBS at 4°C, the resuspended sections were added 10 uL of AnnexinV-FITC and 5 uL of PI staining solution and cultured at room temperature for 15 minutes in the dark. The cells' apoptosis rate was measured by flow cytometry, and the apoptosis rate was calculated by CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA). All experiments were set up in three parallel copies, and the experiments were repeated three times.

Cell migration and invasion capacity detected by Transwell

The migratory and invasive capacities of A549 cells after transfection with si-SFXN1 was measured using Transwell chambers (Corning, Inc.). In invasion assay, Transwell were pre-coated with 8 uM-pore Matrigel (Corning, Inc.). A 200 µL of the pipette was used to add cell suspension into the chambers' interior (upper chamber) with the serum-free medium; meanwhile, 600 µL of DMEM medium containing serum (DMEM complete medium) was added into the lower chamber. The chambers were placed in an incubator with 5% CO₂ at 37°C for 48 hours. After the culture was completed, the cell culture medium in the upper and lower chambers was discarded and washed with PBS buffer twice. After absorbing the residual water with a dry cotton swab, the cells that have not migrated from the membrane's upper surface were removed with a wet cotton swab. Then, cells were stained with a crystalline violet solution for 10-20 minutes. After the staining was completed, the upper chamber was rinsed with PBS buffer, and the upper chamber was dehydrated with xylene for 2-3 minutes and rinsed again with PBS buffer. Finally, the membrane at the bottom of the chamber was cut off with a sterilized scalpel and affixed to a slide. The membrane was placed under a photomicroscope, and five randomly selected fields of view were used for cell counting and photographing.

Statistical analysis

All the data in this study were analyzed using SPSS 19.0 software (IMB Corp.). All experimental data results were expressed as mean \pm standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) or student's t-test to calculate the P values. The association between SFXN1 and clinicopathological features were determined using a χ^2 test. 5-year survival rate and disease-free survival rate were analyzed by Kaplan-Meier survival analysis followed by log-rank. P<0.05 indicated that there was a statistically significant relationship among all results.

Results

SFXN1 was significantly up-regulated in NSCLC tissues and cell lines

We examined the expressions of SFXN1 in NSCLC tissues and cell lines. As shown in Fig. 1A, the SFXN1 level was dramatically increased in NSCLC tumor tissues compared with non-tumor adjacent tissues. Furthermore, data in Fig. 1B verified that SFXN1 expressions were remarkably increased in A549, HCC827, H1299 and NCI-H460 cells related to BEAS-2B cell. Among all, the increasing trend was most significant in A549, which was chosen for the following experiments.

SFXN1 was a feasible diagnostic and prognostic biomarker in NSCLC

First, we analyzed the association between SFXN1 and clinicopathological features. As shown in Table 1, aberrant SFXN1 expression was closely related to lymph node metastasis, tumor size, and TNM stage. However, there were no significant differences in sex, age, smoking, and differentiation. Next, the ROC analysis revealed the high potential of SFXN1 in differentiating NSCLC tissues from non-tumor healthy tissues. As shown in Fig. 2A, the area under the curve (AUC) of SFXN1 was 0.8378 (95% CI = 0.8004 to 0.8751). Furthermore, the Kaplan-Meier analysis in Fig. 2B and 2C revealed that NSCLC patients with higher SFXN1 expression presented unfavorable 5-year survival rate and disease-free survival rate than those with lower SFXN1 expressions.

SFXN1 silencing inhibited A549 cell proliferation, migration, and invasion

To verify the roles of SFXN1 in A549 cells, a si-SFXN1 plasmid was constructed to knockdown SFXN1 expression. The transfection efficiency of SFXN1 in A549 cells was confirmed through q RT-PCR in si-NC, si-SFXN1#, and si-SFXN1* group. As shown in Fig. 3A, SFXN1 levels were remarkably decreased in the si-SFXN1# and si-SFXN1* group; meanwhile, the variance in si-SFXN1# generated the most significant efficiency, which was chosen for the following experiments. After transfection with si-SFXN1# and si-NC into A549 cells, a CCK-8 assay was applied to evaluate the proliferation capability of A549 cells. Fig. 3B showed that SFXN1 knockdown significantly inhibited A549 cell proliferation compared with si-NC or control group. As for measuring the metastasis of A549 cells, cell migration and invasion capabilities were evaluated by wound healing and Transwell assays, respectively. As shown in Fig. 3C-F, we found that silencing of SFXN1 prevented the migratory and invasive rates of A549 cells.

Knockdown of SFXN1 promoted A549 cell apoptosis

We examined the effects of SFXN1 silencing on A549 cell apoptosis by flow cytometry analysis. As shown in Fig. 4A-4D, compared with si-NC or control group, the cell apoptosis rate was obviously increased after transfection with si-SFXN1#, suggesting that SFXN1 may have an anti-apoptotic role in A549 cells.

Silencing of SFXN1 decreased PCNA, Bcl-2, MMP2, and MMP-9 levels while increased Bax level in A549 cells

PCNA, Bcl-2, Bax, MMP2, and MMP9 played crucial roles in NSCLC cell proliferation, apoptosis, and metastasis. Therefore, we detected the effects of SFXN1 silencing on A549 cell growth-related proteins. As shown in Fig. 5A-5B, SFXN1 silencing markedly reduced PCNA and Bcl-2 while increased Bax level related to cancer proliferation and apoptosis. Moreover, MMP2 and MMP9 levels were significantly inhibited while transfected with si-SFXN1# in A549 cells compared with si-NC or control group.

Discussion

This study first found that SFXN1 levels dramatically increased in NSCLC tumor tissues and cell lines. Furthermore, TNM stage, tumor size, and lymph node metastasis were verified to be correlated with SFXN1 expression. Meanwhile, ROC and Kaplan-Meier survival analysis confirmed the potentials of SFXN1 in NSCLC diagnosis and prognosis. In vitro experiments, after constructing SFXN1 silencing plasmid, functional studies helped illustrate the oncogenetic role of SFXN1, where SFXN1 knockdown impaired A549 cell proliferation, migration, and invasion while promoted cell apoptosis ability. These studies implied that SFXN1 might be a feasible biomarker for NSCLC diagnosis and prognosis.

Apoptosis was a form of programmed cell death that occurred in multicellular organisms [9]. Cell apoptosis was thought to be an essential component of a variety of processes; meanwhile, abnormal apoptosis was a crucial factor in many human diseases, including autoimmune diseases [10, 11] and many cancers [12–14]. The mechanisms of apoptosis were complex which involved energy-dependent molecular cascade reactions [9]. To date, studies have shown that there were two main apoptotic pathways: the exogenous or death receptor pathway and the endogenous or mitochondrial pathway [15]. Of these, the mitochondrial pathway was regulated by members of the Bcl-2 protein family, which controlled the mitochondrial membranes' permeability [16–18]. Recently, a total of 25 genes have been identified in the Bcl-2 family, including anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax [19]. In 1990, the Bcl-2 protein was reported to induce cancer development by limiting cell death [20]. Also, many studies confirmed the anti-apoptotic role of Bcl-2 in NSCLC as well [21, 22]. Bax was abundantly expressed in hepatocytes, renal tubular epithelial cells, and bronchial smooth muscle and vascular smooth muscle cells, exerting the protective effect of Bcl-2 at the cellular level [23]. Bax's pro-apoptotic role was verified in various cancers, such as breast cancer [24], prostate cancer [25], and NSCLC [26]. Consistent with previous studies, our results found that silencing of SFXN1 inhibited PCNA and Bcl-2 levels while increased Bax level, thereby reduced A549 cell proliferation but promoted apoptosis.

Multiple studies discovered that MMP2 and MMP9, two metastasis-related proteins, were widely expressed in NSCLC [27, 28]. MMP2 and MMP9 belonged to the MMP gene family, which was considered to be involved in multiple pathways, including embryonic development, reproduction, tissue remodeling, arthritis, and metastasis [29]. Jin et al. [30] and Li et al. [31] reported that inhibition of A549 cell migration and invasion might be related to being the decrease in MMP2 and MMP9 expressions. In terms of previous studies, we measured the levels of MMP2 and MMP9 after knocking down SFXN1 expression. As expected, A549 cell migration and invasion were hindered along with decreased MMP2 and MMP9 expressions.

SFXN1, a mitochondrial serine transporter, functioned as a multipass inner mitochondrial membrane protein [32]. Previous reporters demonstrated that diseases associated with SFXN1 included neonatal anemia and insulin resistance [33, 34]. Huang et al. [35] reported that SFXN1 was significantly increased in synovitis of osteoarthritis. Another study also illustrated that SFXN1 was dysregulated in lung adenocarcinoma patients, associated with poor prognosis [36]. Our research firstly displayed and validated the underlying mechanisms of SFXN1 in NSCLC diagnosis, prognosis, and development. Through χ^2 test between aberrant SFXN1 expressions and clinicopathological parameters, we verified

that SFXN1 expression was strongly correlated with lymph node metastasis, tumor size, and TNM stage in NSCLC.

Throughout the text, we should recognize that this retrospective study has several limitations: i) all NSCLC patients enrolled in this study received surgical resection; however, they were not classified into different groups according to TNM stage; ii) the sample size of this study was relatively small. Considering that the clinical samples were collected at the same hospital, a more extensive and multicenter study must be conducted in the future; iii) A549 cell line was used for the in vitro experiments in this paper, and no in vitro experiments were performed; iv) the expression of SFXN1 may be different in different cancers and at various stages of cancer development. More work is needed in the future better to understand the expression and prognosis of SFXN1 in tumors.

Conclusion

In sum, these data suggested that SFXN1 may differentiate NSCLC patients and predict clinical outcomes and exert as a potential candidate for the NSCLC treatment target.

Declarations

Funding

None.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Affiliated Huaian No.1 People's Hospital of Nanjing Medical University between Ethics Committee (No. 201302875).

Consent for publication

All authors reviewed and approved the final version of the manuscript and consented for publication.

Competing interests

The authors declare that they have no Competing interests.

Author's Contribution

XZN designed the research/study. HZ and LYG performed the research/study and wrote the manuscript. XZN, LYG and CS collected the data. HZ and CS analyzed the data. All authors read and approved the final manuscript.

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None.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

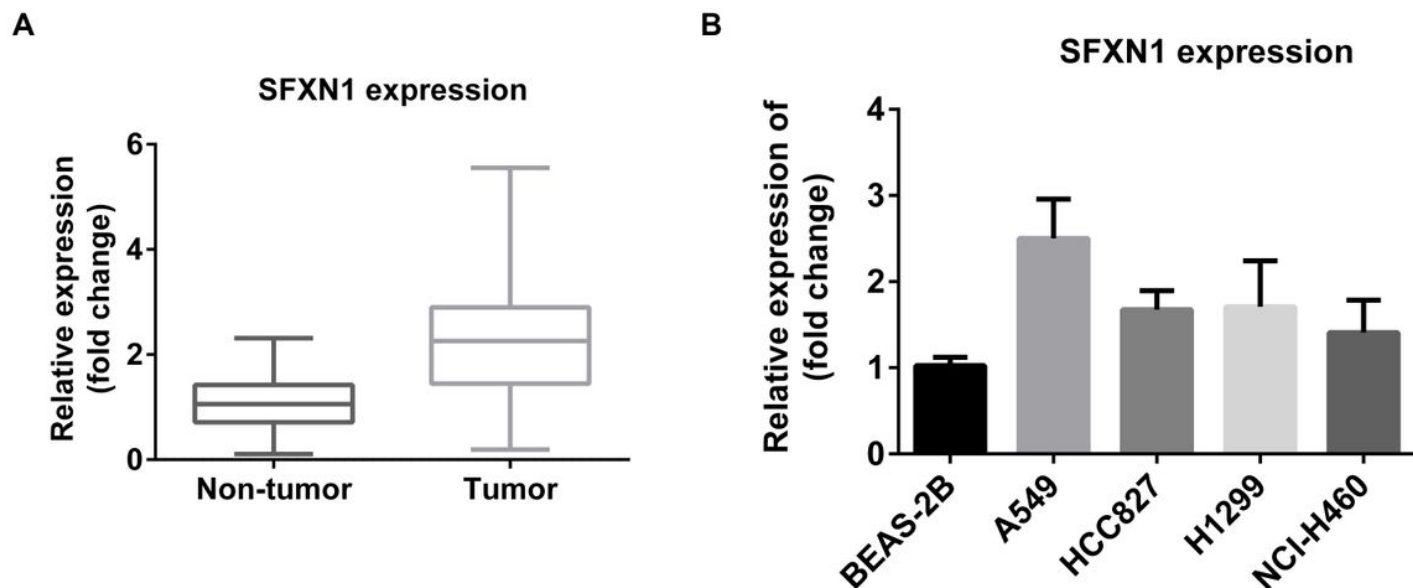


Figure 1

SFXN1 was dramatically up-regulated in NSCLC. (A) The SFXN1 level in NSCLC tumor and non-tumor adjacent tissues were assessed by qRT-PCR assay. (B) Relative expressions of SFXN1 in BEAS-2B and NSCLC cell lines (A549, HCC827, H1299, and NCI-H460) were detected by the qRT-PCR assay. NSCLC, non-small cell lung cancer; SFXN1, sideroflexin 1.

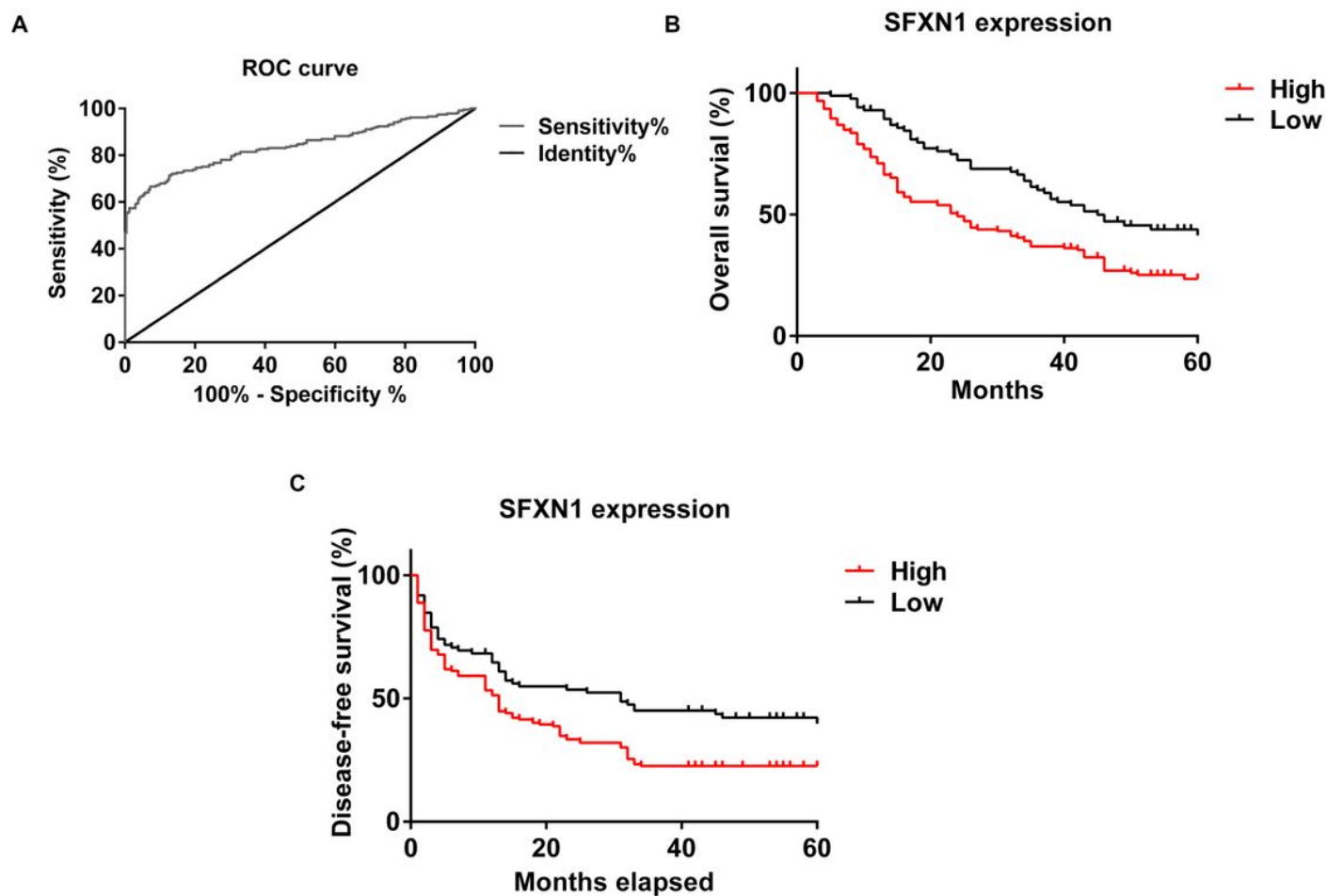


Figure 2

The potentials of SFXN1 in NSCLC diagnosis and prognosis. (A) ROC analysis revealed the predictive role of SFXN1 in differentiating tumor tissues from non-tumor adjacent tissues in NSCLC. (B) The 5-year survival rate of all NSCLC patients with abnormal SFXN1 expressions. (C) The disease-free survival rate of all NSCLC patients with aberrant SFXN1 levels. SFXN1, sideroflexin 1.

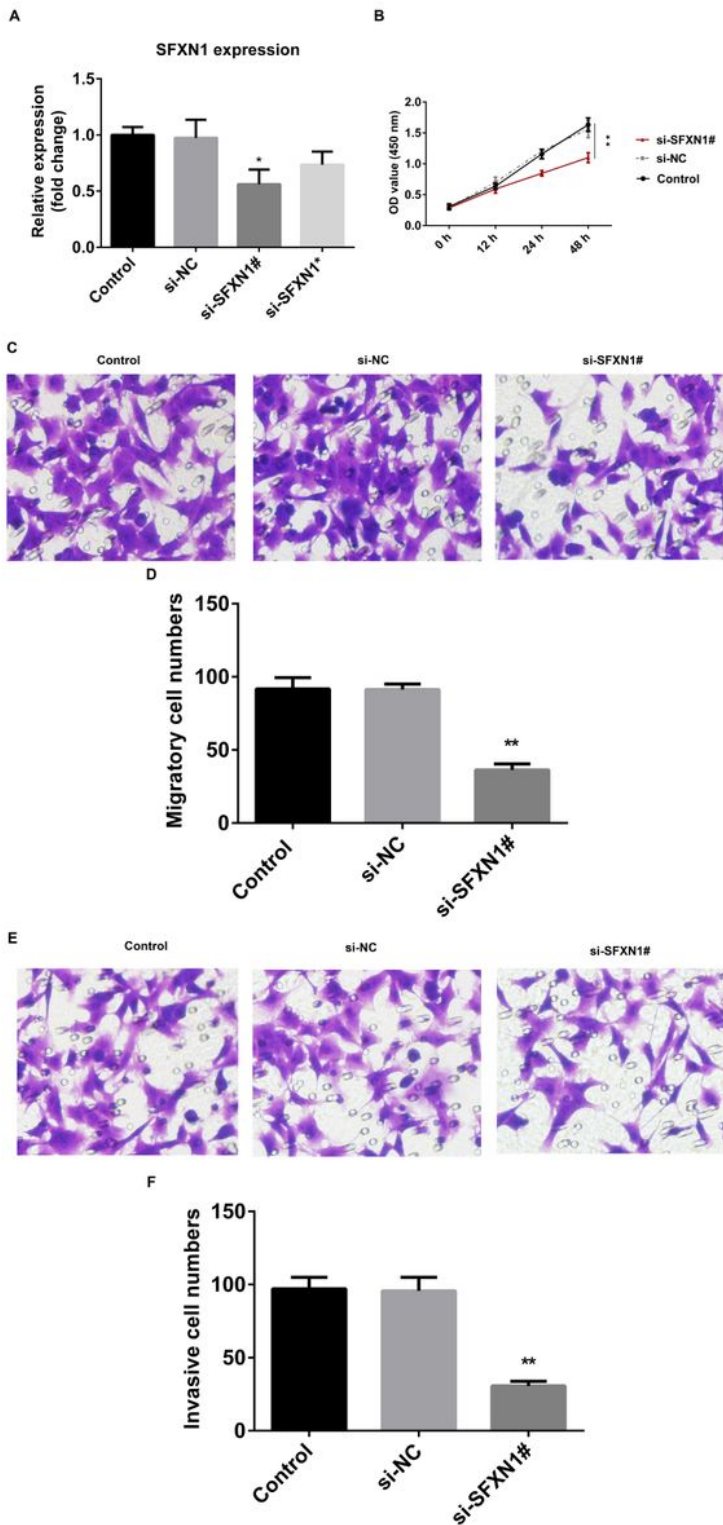


Figure 3

SFXN1 silencing significantly inhibited A549 cell proliferation, migration, and invasion. (A) The transfection efficiency of SFXN1 knockdown in A549 cells was confirmed by qRT-PCR assay. (B) Knockdown of SFXN1 notably reduced A549 cell proliferation. (C-D) Silencing of SFXN1 remarkably decreased A549 cell migration. (E-F) SFXN1 silencing prominently inhibited A549 cell invasion. **P<0.01, si-SFXN1# vs. si-NC group. SFXN1, sideroflexin 1.

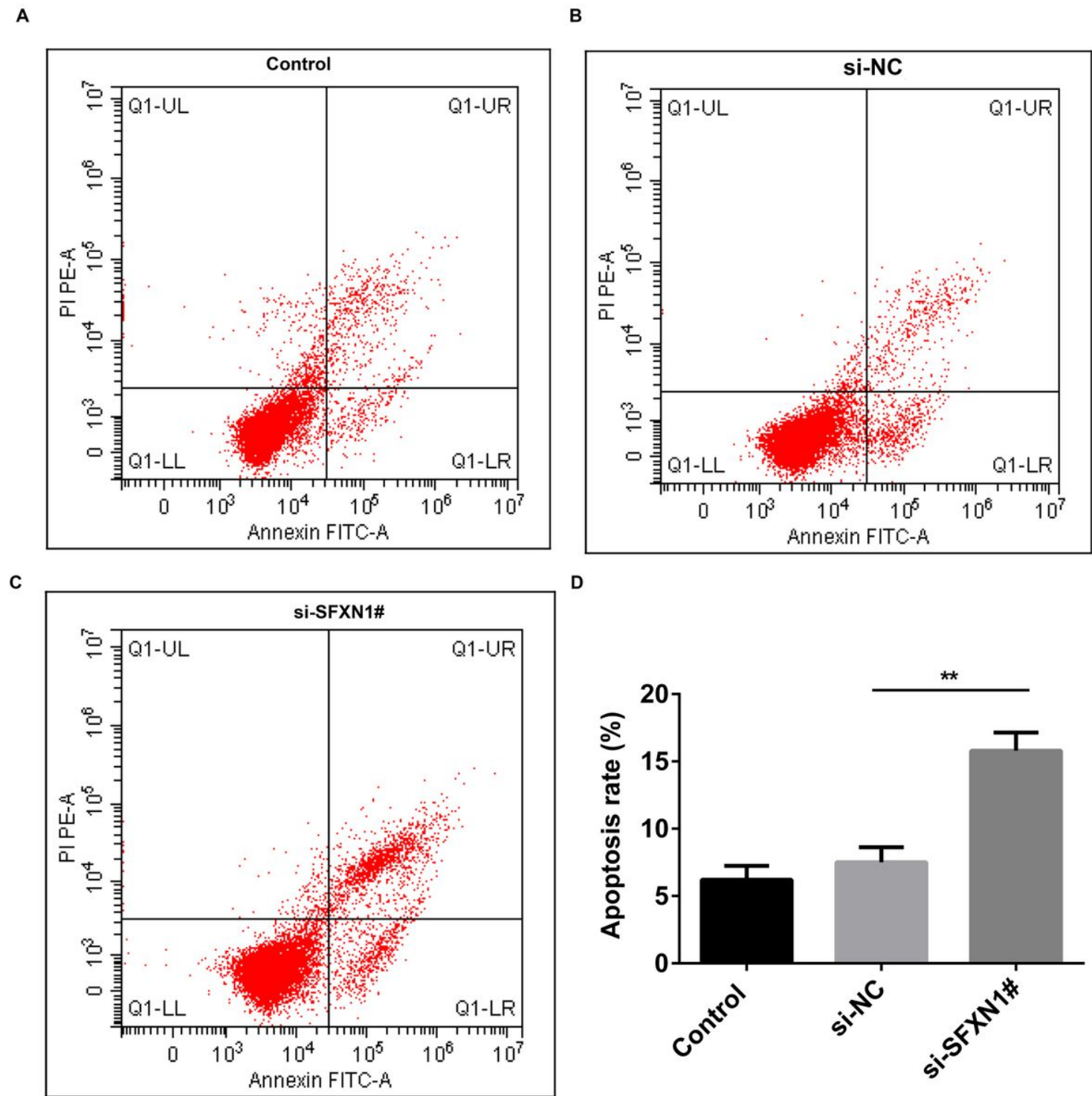


Figure 4

Knockdown of SFXN1 apparently induced A549 cell apoptosis. (A) Control group. (B) si-NC group. (C) si-SFXN1# group. (D) Quantified results of A-C were displayed. $**P<0.01$, si-SFXN1# vs. si-NC group. SFXN1, sideroflexin 1.

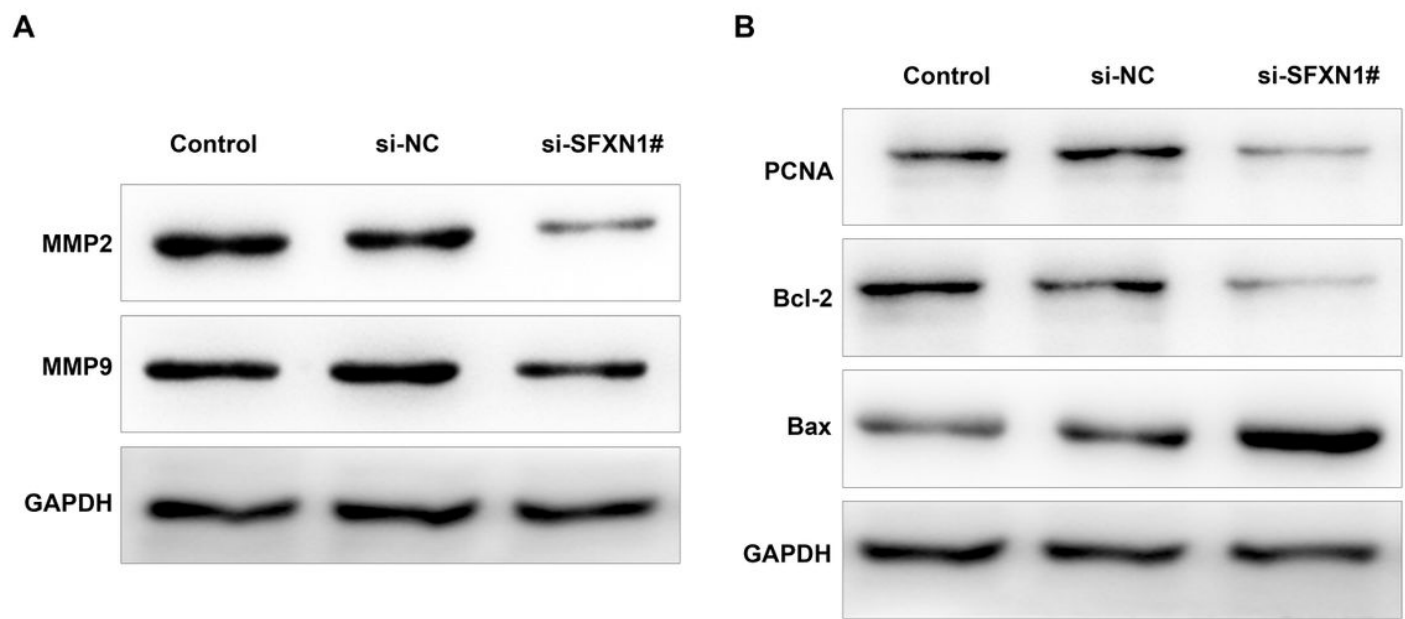


Figure 5

The effects of SFXN1 silencing on tumor growth and metastasis-related proteins PCNA, Bcl-2, Bax, MMP2, and MMP9 in A549 cells. (A) Protein bands of MMP2 and MMP9. (B) Protein bands of PCNA, Bcl-2 and Bax. PCNA, proliferating cell nuclear antigen; Bcl-2, b-cell lymphoma-2; Bax, BCL-2-associated X protein; MMP2, matrix metalloproteinase 2; MMP9, matrix metalloproteinase 9; GAPDH, glyceraldehyde-phosphate dehydrogenase.