

Knockdown of CHPF suppresses cell progression of non-small-cell lung cancer

Xiao-Ming Hou^{1,2,*}
 Zulqarnain Baloch^{3,4,*}
 Zhan-Hong Zheng^{3,4}
 Wen-Hui Zhang^{1,2}
 Ying Feng⁵
 Duan-Duan Li^{3,4}
 Xin-An Wu^{2,6}
 Shi-Hua Yang^{3,4}

¹Department of Oncology, The First Hospital of Lanzhou University, Lanzhou 730000, People's Republic of China; ²Key Laboratory of Biotherapy and Regenerative Medicine of Gansu Province, The First Hospital of Lanzhou University, Lanzhou 730000, People's Republic of China; ³College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, People's Republic of China; ⁴Key Laboratory of Comprehensive Prevention and Control for Severe Clinical Animal Diseases of Guangdong Province, South China Agricultural University, Guangzhou 510642, People's Republic of China; ⁵Department of Pathology, The First Hospital of Lanzhou University, Lanzhou 730000, People's Republic of China; ⁶Department of Pharmacy, The First Hospital of Lanzhou University, Lanzhou 730000, People's Republic of China

*These authors contributed equally to this work

Correspondence: Xin-An Wu
 Key Laboratory of Biotherapy and Regenerative Medicine of Gansu Province, First Hospital of Lanzhou University, Lanzhou 730000, People's Republic of China
 Tel +86 931 861 6392
 Fax +86 931 861 6392
 Email ldywx@163.com

Shi-Hua Yang
 College of Veterinary Medicine, South China Agricultural University, 483 Wushan Road, Tianhe District, Guangzhou 510642, People's Republic of China
 Tel +86 208 528 7341
 Fax +860 208 528 0234
 Email yangsh@scau.edu.cn

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Purpose: The aim of the present study was to explore the role of CHPF in non-small-cell lung cancer (NSCLC) and to develop an shRNA vector-based therapy to repress the expression of CHPF gene in NSCLC cell lines.

Methods: In this study, we used immunohistochemical staining to verify the expression of CHPF in NSCLC tissue. Then, we determined the expression of CHPF gene in different NSCLC cell lines with RT-PCR and Western blotting. Specific CHPF shRNA was used to knockdown the expression of CHPF. Celigo image cytometry, cell cycle analysis, and flow cytometry assay were performed.

Results: The results showed that expression level of CHPF was higher in NSCLC tissues than normal lung tissues. Further, we established that CHPF expression knockdown in NSCLC cells could substantially restrain the cell proliferation, apoptosis, and cell cycle in vitro.

Conclusion: On the basis of these results, we concluded that CHPF expression has an important role in the progression of human NSCLC cells. Therefore, its interference could possibly be used as a potential therapeutic target against NSCLC.

Keywords: NSCLC, CHPF, progression, knockdown

Introduction

Lung cancer is a major cause of cancer-related death globally.¹ According to WHO estimates, lung cancer constitutes 13% of total cancer incidence and was responsible for 19.4% of cancer mortality in 2012.^{2,3} Among them, most lung cancers are non-small-cell lung cancer (NSCLC). Despite the tremendous progresses in diagnostic and therapeutic methods in recent years, NSCLC still remains the foremost reason of cancer-related morbidity and mortality in the world. The malignant biological behavior of cancer, particularly NSCLC, is regulated by multiple genes, the occurrence and increased tumorigenesis of NSCLC is associated with the loss of activity of tumor-suppressor genes and high expression of tumor-related genes.

Chondroitin sulfate (CS) is found naturally in the body and consists of a chain of alternating sugars (N-acetylgalactosamine and glucuronic acid).⁴ Biosynthesis of CS is instigated by the adding of xylose to serine residues in a core protein, followed by the chronological addition of two residues, Gal and single residue of GlcA, to develop the tetrasaccharide linkage structure *GlcAb1-3Galb1-3Galb1-4Xylb1-O-Ser*.⁵ The synthesis of CS requires the promotion of molecules such as *CHPF*. Until now, four genes, *CHSY1*, *CHPF*, *CHSY3*, and *CHPF2*, have been identified to play an important role in CS biosynthesis. It has also been reported that each combination of these four enzymes is also able to control CS biosynthesis.⁶ In

recent publications, the relationship between *CHPF* genes and human diseases has been explored.^{7,8} *CHPF* is a kind of protein-coding gene. Kitagawa et al isolated *CHPF* by catalog-probing with the *CHSY1* sequence.⁵ *CHPF* gene has at least four exons over 5 kb in length. *CHPF* consists of 775-amino-acid protein with approximately 85 kD molecular mass and encloses three potential N-glycosylation locations. The sequence *CHPF* gene has important N-terminal hydrophobic sections and is envisaged a type II trans-membrane protein.⁵ *CHPF* gene has 23% and 57% similar sequence identity with human *CHSY1* and *CHPF2*, respectively.⁶

RNAi is a significant technological advancement in the field of modern biology, and has been widely used in *in vitro* and *in vivo* experiments to effectively knockdown target genes' expression.⁹ siRNAs have already been effectively used in different types of mammalian cell lines, and have successfully caused 80% downregulation of candidate gene expression.¹⁰ siRNAi technology provides a short-term suppression of target gene expression. Therefore, alternative approaches have been introduced such as shRNAs, to obtain long-term suppression of target gene expression.¹¹

Recently, it has been verified that *CHPF* expression plays an important role in initiation and development of colorectal cancer.⁷ However, *CHPF* expression's role in the progression of NSCLC remains unclear. Therefore, in this study, we examined the role of *CHPF* in NSCLC and then designed shRNA-*CHPF*-Lenti vector to knockdown the *CHPF* gene in NSCLC cell lines. At the end we explored the effect of the knockdown of *CHPF* in NSCLC cell lines on cell proliferation.

Methods

Tissue specimens

A total of 90 pairs of NSCLC tissues and adjacent tissues were obtained from The Hospital of Lanzhou University. All patients were requested to sign informed consent. No patient received any prior chemotherapy or radiotherapy. The protocols were approved by the Ethics Committee of The Hospital of Lanzhou University.

Immunohistochemical staining and evaluation

Formalin-fixed paraffin-embedded tissue block was incised into 4 μ m thick sections. All incised sections were deparaffinized with xylene and rehydrated with graded

alcohol. After incubating with 3% H₂O₂ for 20 minutes at room temperature to satiate the activity of endogenous peroxidase, the sections were further processed in sodium citrate buffer (pH =6.0) for heat-induced antigen retrieval. After washing several times, the pieces were incubated in 10% goat serum at room temperature for 30 minutes to block nonspecific binding. Then, the slides were incubated with primary anti-*CHPF* (Santa Cruz Biotechnology Inc., Dallas, TX, USA) antibody at 4°C overnight. After the primary antibody was washed off, anti-rabbit peroxidase-conjugated secondary antibody and DAB reagent were employed in the detection procedure.

Two qualified, independent pathologists who were blinded to the clinical and pathological data of the tumors. The staining of slides was observed and photographed using an Olympus microscope system (Olympus Corporation, Tokyo, Japan). The percentages of positive tumor cells were semi-quantitatively graded as 0 (<5%), 1 (5%–25%), 2 (26%–50%), and 3 (>50%). The staining intensity of tumor cells was scored according to criteria reported previously. The two scores were multiplied to acquire the final immunoreactive score. High expression of *CHPF* in tumor cells was defined as immunoreactive scores ≥ 4 .

Cell culture

The human NSCLC cell lines H460, H1299, H1688, 95-D, and A549 were purchased from the Shanghai Branch of the Chinese Academy of Sciences. All cells were grown in DMEM supplemented with 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) in a 37°C incubator with 5% CO₂. In this study, transfection was performed using Genechem Transfection Reagent (Genechem Co., Ltd., Shanghai, People's Republic of China) according to the manufacturer's instructions.

RNA extraction and RT-PCR

In this study, total RNA was extracted with Trizol (Invitrogen, Thermo Fisher Scientific) by following the manufacturer's instructions. An ultraviolet spectrophotometer was used to measure the concentration. An amount of 5 μ g RNA was reverse transcribed by PrimeScript™ RT reagent Kit (Takara, Japan). according to the manufacturer's instructions. We used Premier Primer 5.0 software to design the PCR primers. The primer sequences are shown in Table 1. We used SYBR_Green PCR Master Mix™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) to perform RT-PCR. The PCR conditions were 30 minutes at 37°C, 5 minutes at 83°C.

Table 1 Primers used in this study for PCR

Primers	Sequence
<i>CHPF</i> forward primer	GGAACGCACGTACCAGGAG
<i>CHPF</i> reverse primer	CGGGATGGTGTGGAATACC
<i>GAPDH</i> forward primer	TGACTTCAACAGCGACACCCA
<i>GAPDH</i> reverse primer	CACCCTGTTGCTGTAGCCAAA

PCR was performed in 10 μ L reactions with the following reaction conditions: 2 minutes pre-denaturation at 95°C, 30 cycles of 15 seconds denaturation at 95°C, 20 seconds at 60°C. The relative gene expression levels were measured and compared by $2^{-\Delta\Delta CT}$ analysis program.

Lentiviral vector and cell transfection

The shRNA against *CHPF* (NM_024536; GenBank) gene was cloned into lentiviral gene transfer vector with GFP (Genechem Co., Ltd.). The sequence of *CHPF* shRNA was 5'-CTGGCCATGCTACTCTTTG-3'. The non-targeted control mock lentivirus (LV-NC virus) and recombinant *CHPF* targeting lentivirus (LV-*CHPF*-shRNA virus) were made and transfected according to the manufacturer's instructions for NSCLC cell lines. In short, after the lentivirus was constructed, 293T cells were seeded in 6-well plates and cells were infected to test its efficacy when cell growth reached 70%–80% confluency. After 24, 48, and 72 hours' transfection, viruses were collected. The infected cells were named shCtrl group and shCHPF group, respectively.

For lentivirus transfection, NSCLC cells were cultivated in 6-well plates and negative control (shCtrl) lentivirus or the *CHPF*-shRNA-lentivirus was dropped according to multiplicity of transfection. Cultured cells were inspected using a fluorescence microscope (MicroPublisher 3.3; Olympus) after 72 hours of transfection. The cells were collected to determine the knockdown efficiency by quantitative RT-PCR.

Western blotting

Total protein was extracted from cells by lysing in RIPA buffer. The total cell protein extract was determined and measured with BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, People's Republic of China). Equal amounts of cell lysate were separated on 10% SDS-PAGE (Bio-Rad Laboratories Inc., Hercules, CA, USA) and transferred to a PVDF membrane (EMD Millipore, Billerica, MA, USA). The PVDF membrane was blocked with Tris-buffered saline/Tween with 5% non-fat milk for 2 hours at room temperature or overnight at 4°C and incubated with

primary antibody and then with secondary antibody. ECL Western Blotting Substrate kit (Pierce, Thermo Fisher Scientific) was used to visualize protein bands. We purchased the primary anti-flag antibody from Sigma Biotechnology (Sigma-Aldrich Co., St Louis, MO, USA) and mouse anti-*GAPDH* from Santa Cruz Biotechnology Inc. Goat anti-mouse IgG and the secondary antibody was purchased from Santa Cruz Biotechnology Inc.

Cell growth assay

Cells in logarithmic growth phase were digested with trypsin (Sangon Biotech, Shanghai, People's Republic of China) and resuspended. Then, the cells were seeded in a 96-well plate at a density of 2,000 cells/100 μ L/well. The cells were incubated overnight at 37°C and 5% CO₂. From the second day after plating, we continuously measured the number of cells over 5 days using a Celigo image cytometer (Nexcelom Bioscience, Lawrence, MA, USA). Statistics were performed on the data to plot the proliferation curve of the cells. The cell proliferation ratio was calculated based on the cell count and the time-point, and a growth curve based on the cell proliferation factor was drawn.

Cell cycle assay

The NSCLC cells from sh*CHPF* and shCtrl groups were inoculated in 6-well plates and cultured for 48 hours. Next, we used ice-cold PBS to wash the cells and fixed them in a 70% (v/v) ice-cold ethanol solution at 4°C overnight. Then, we used flow cytometry (EMD Millipore) to analyze the cells.

Cell apoptosis was analyzed using the Annexin V-allophycocyanin kit (Annexin V-APC; Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were collected and processed as previously mentioned. The cell concentration was adjusted to 1×10^6 /mL by using staining buffer. Finally, 100 μ L of suspension was stained with 5 μ L Annexin V-APC.

Human apoptotic protein array

Human Apoptosis Antibody Array kit (Ray-Biotech, GA, USA) was used to perform apoptosis array test. NSCLC cells treated with shRNA were seeded in 6-well plates for 72 hours. Treated cells were collected after 72 hours and centrifuged at 2,500 rpm, for 5 minutes. Collected cells were washed twice using ice-cold PBS. We again performed centrifugation for 5 minutes at 2,500 rpm, then the supernatant was discarded. Cell protein extraction was completed and about 500 mg extracted proteins were incubated with the

human apoptosis array overnight. Chemiluminescence detection was performed by scanning the membrane on Odyssey FC Imaging System (LI-COR, USA).

Statistical analysis

Statistical analysis was performed with SPSS 19.0 program (IBM Corporation, Armonk, NY, USA). One-way ANOVA was applied for comparisons between multiple groups and the Student–Newman–Keuls method was used for comparisons between two groups. All data were presented as mean \pm SD, and $P < 0.05$ was considered statistically significant.

Results

CHPF is highly expressed in NSCLC

We determined the expression of *CHPF* in NSCLC tissues using immunohistochemistry. We found that *CHPF* protein was mainly localized in the cytoplasm of cells (Figure 1). Of the 90 NSCLC samples, the expression rate of *CHPF* in NSCLC tissues was 55.6% (50/90), which was significantly higher than that in adjacent tissues (28/90; $P = 0.001$). According to immunohistochemistry results, 90 patients with NSCLC were divided into *CHPF* low expression and high expression groups. At the same time, we analyzed the relationship between clinicopathological features and *CHPF* expression in patients with NSCLC. The results suggest that high expression of *CHPF* is associated with tumor size and TNM stage in NSCLC patients (Table 2).

CHPF mRNA expression was determined in the following cell lines: H460, H1299, H1688, 95-D, and A549 by RT-PCR (Figure 2A). The *CHPF* protein expression level was detected in these cell lines by using Western blotting (Figure 2B). Our observation indicated that *CHPF* mRNA expression was high in all A549 cell lines. Therefore, we used A549 cell lines for subsequent functional experiments.

Knockdown efficiency determined by Western blot analysis

To explore the role of *CHPF*, we constructed the sh*CHPF* with recombinant lentivirus and transfected the sh*CHPF* into NSCLC A549 cell lines. Then, the transfection efficiency was observed by fluorescence microscope. The percentage of infected cells was $>80\%$ for shCtrl and sh*CHPF* lentivirus 3 days post-transfection (Figure 3). A549 cell lines were infected with *CHPF*-shRNA or shCtrl lentivirus. In order to verify the efficiency of knockdown, the expression of *CHPF* protein was determined by Western blotting. We found that the expression of *CHPF* protein was decreased as compared to negative control (Figure 4).

Knockdown of *CHPF* inhibits cell proliferation

In this study, we analyzed the effect of *CHPF* expression on NSCLC cell growth by Celigo image cytometer for 5 days. The results showed that control cells' growth increased during 5 days, while growth of *CHPF*-shRNA-transfected cells decreased during the experiment period (Figure 5). It is suggested that the *CHPF* gene is significantly associated with the proliferative capacity of A549 cells.

Effect of *CHPF* on cell cycle and cell apoptosis

Three days after shRNA lentivirus infection, compared with the shCtrl group, the number of cells in the G1 phase of sh*CHPF* group A549 cells increased, the number of cells in the S phase decreased, and the number of cells in the G2/M phase decreased (Figure 6). It is suggested that the *CHPF* gene is significantly associated with the periodic distribution of A549 cells.

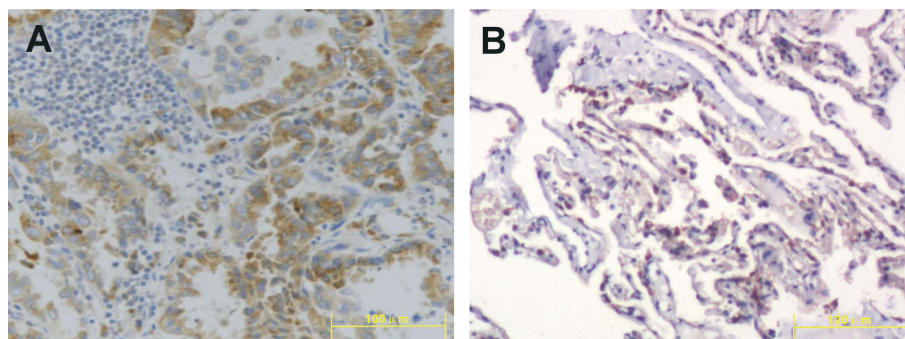


Figure 1 Expression of *CHPF* in non-small-cell lung cancer tissue (A) and adjacent tissue (B) $\times 200$.

Table 2 The correlations between *CHPF* expression and clinicopathological features in NSCLC patients

Variables	Cases	<i>CHPF</i> expression		P-value
	(n=90)	Low (n=40)	High (n=50)	
Sex				0.627
Male	56	26	30	
Female	34	14	20	
Age (years)				0.912
≤65	55	24	31	
>65	35	16	19	
Smoking				0.257
Yes	48	24	24	
No	42	16	26	
Histological type				0.549
Adenocarcinoma	60	28	32	
Squamous cell carcinoma	30	18	34	
Differentiation				0.665
Well/moderate	54	25	29	
Poor	36	15	21	
Tumor size (cm)				0.028
≤3	38	22	16	
>3	52	18	34	
LN metastasis				0.061
Negative	58	30	28	
Positive	32	10	22	
TNM stage				0.003
I	18	14	4	
II	32	14	18	
III	40	12	28	

Abbreviations: NSCLC, non-small-cell lung cancer; LN, lymph node.

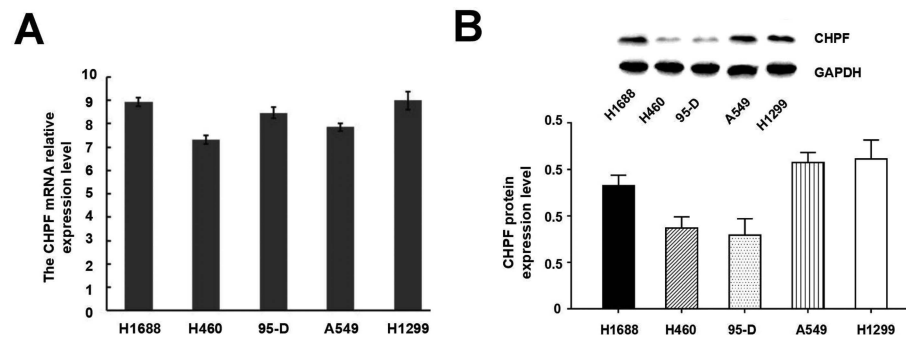


Figure 2 Expression of *CHPF* in five non-small-cell lung cancer cell lines. **(A)** *CHPF* mRNA was detected by RT-PCR; **(B)** *CHPF* protein was detected by Western blotting.

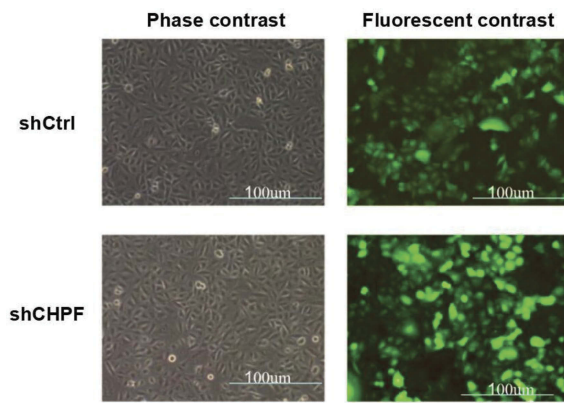


Figure 3 Observation of the transfection efficiencies using fluorescence microscopy.

After 4 days of shRNA lentivirus infection, compared with the shCtrl group, the apoptosis of A549 cells in the shCHPF group increased significantly (Figure 7). The results suggested that *CHPF* gene is significantly associated with apoptosis of A549 cells.

Human apoptotic protein array

We used human apoptotic protein array to determine the semi-quantitative of apoptotic proteins in A549 cells. The concentration of signal for antigen-specific antibody spot was comparative to the antigen-relative concentration. Further, we observed that the positive control signal on all array images had comparable intensities. Therefore, evaluation of signal intensities for every antigen-specific antibody spot between the array membranes might be applied to count the relative protein expression differences. In A549 cells, the expression level of Caspase-3 protein was upregulated after silencing *CHPF* by RNAi, and other proteins showed no significant changes (Figure 8).

Discussion

During past decades, researchers have mainly concentrated on discovering potential biomarkers which can be used for treatment and diagnosis of cancers. However, the clinical applications of those molecular markers are very restricted. Therefore, scientists are continually concentrating on the identification of new potential genes. Exploring new potential candidate sites and understanding the related signaling pathways is very important. It has been previously confirmed that many genes such as *EGFR*, *ALK* or *ROS1* have very important roles in the initiation of tumorigenesis and development of cancers, particularly NSCLC.^{12–14} RNAi technology has been widely used for gene silencing.¹¹ The application of RNAi technology in cancer treatment has already been proven in in vitro and in vivo model experiments.¹⁵ In this study, shRNA technology effectively silenced the expression of *CHPF* in the NSCLC cell lines, which laid an important foundation for subsequent research.

Here, we have effectively knocked down the expression of *CHPF* gene in A549 cell lines by using RNAi method, and further, we examined the effect of *CHPF* expression knock-down on the regulation of cell proliferation, cell cycle, and apoptosis. According to the best of our knowledge, nobody has investigated the role of *CHPF* expression in NSCLC. At first we investigated the expression of *CHPF* in human NSCLC cell lines and tissues. We observed that *CHPF* expression was substantially increased in the NSCLC tissues from patients, compared to adjacent tissues. The elevated expression of *CHPF* supported that *CHPF* might contribute to proliferation of NSCLC. Our observation is consistent with results of Kalathas et al, in which they reported that *CHPF* expression was increased in colorectal cancer tissues compared to normal tissue.⁷ Therefore, a detailed study of sulfotransferases, glycosyltransferases, and dermatan sulfate

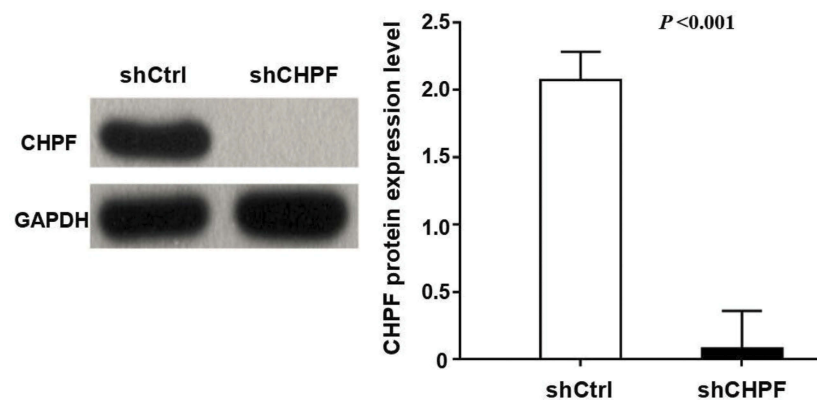


Figure 4 Western blotting-verified transfection efficiency of A549 cell lines.

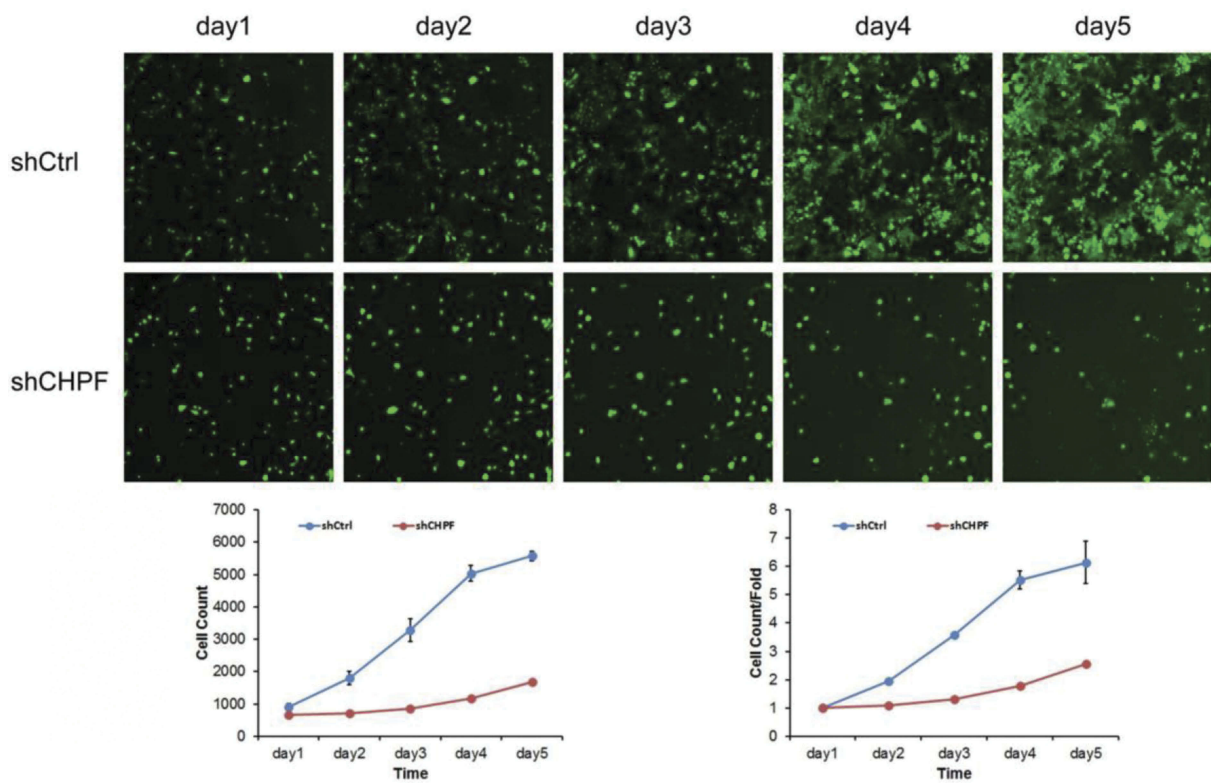


Figure 5 Cell proliferation ability determined by Celigo assay. The proliferation of A549 cells was significantly inhibited after knockdown of *CHPF*.

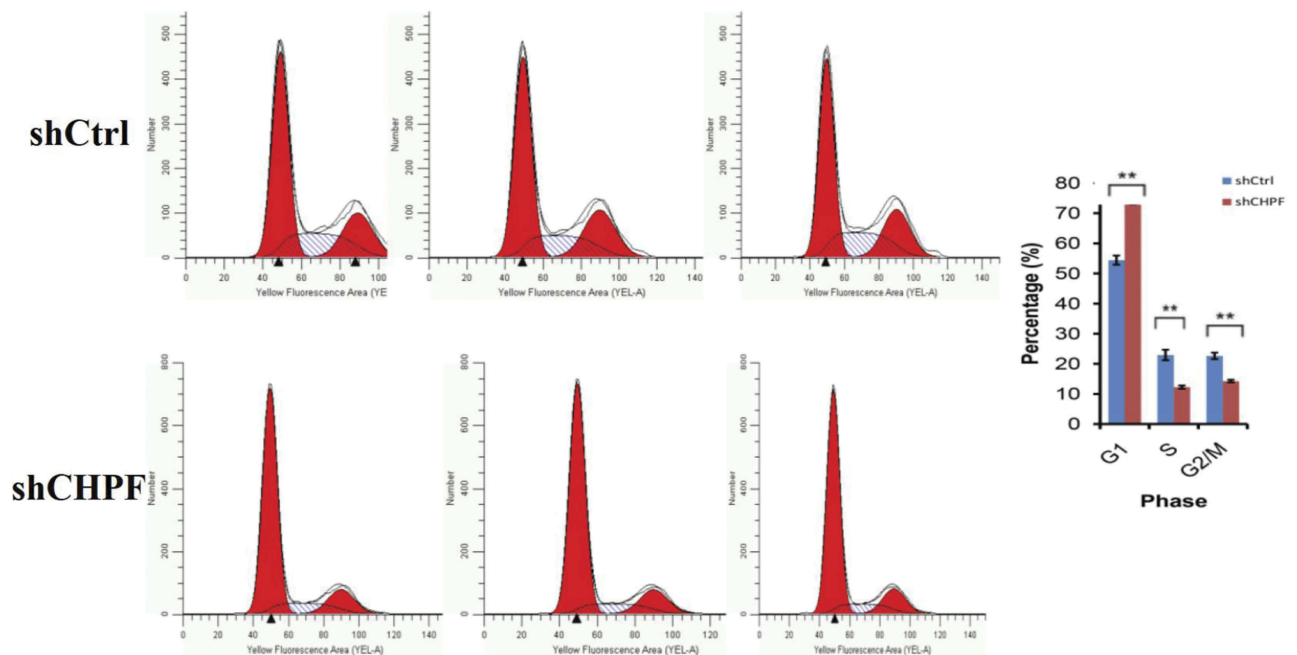


Figure 6 Cell cycle of A549 cell lines was examined by flow cytometry. The A549 cells were arrested in G1 phase after knockdown of *CHPF*.

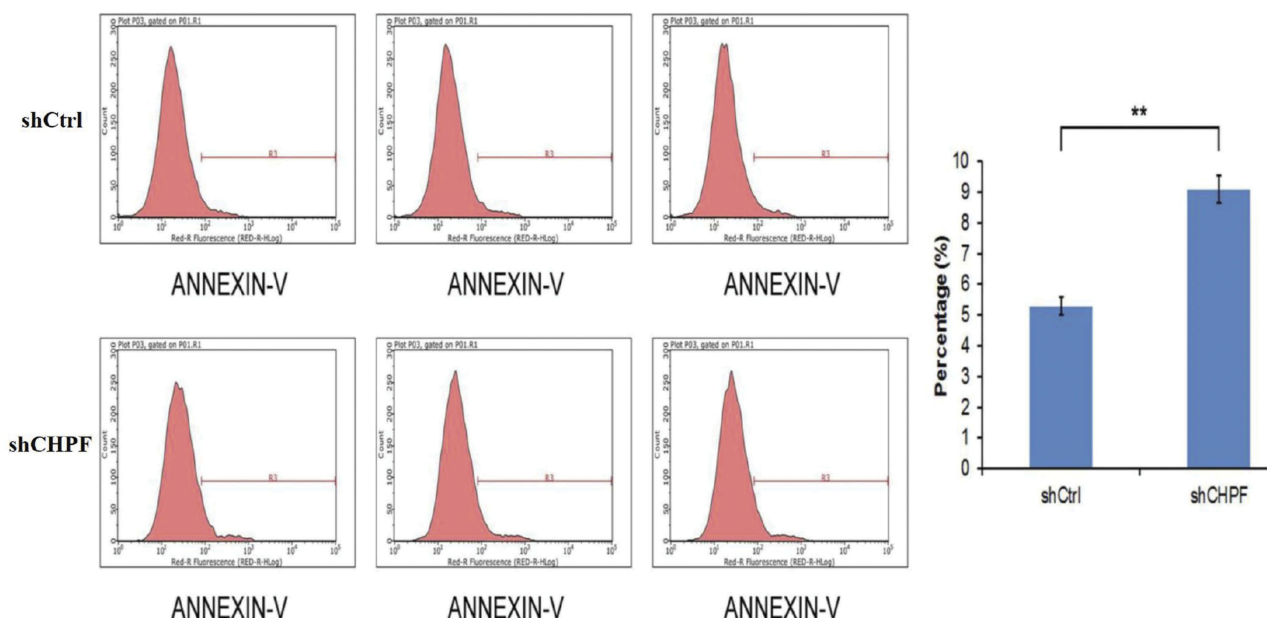


Figure 7 Analysis of apoptosis of A549 cell lines using Annexin V-allophycocyanin kit. The apoptosis of A549 cells was significantly increased after knockdown of *CHPF*.

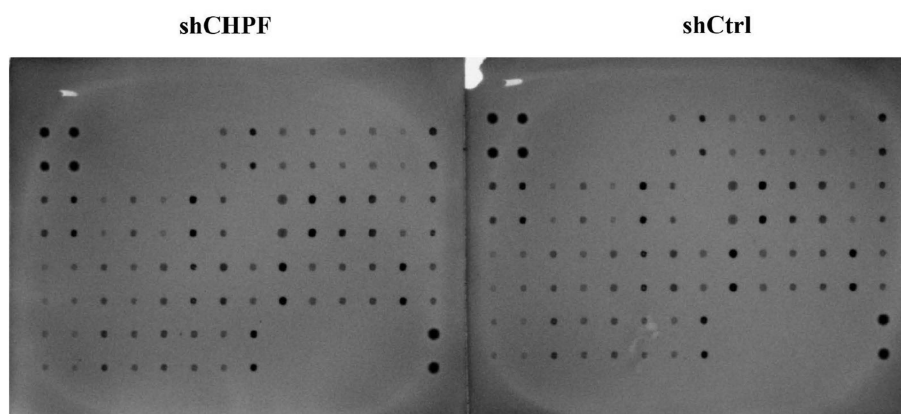


Figure 8 The human apoptotic protein array was used to determine apoptotic proteins. The Caspase-3 protein was upregulated after knockdown of *CHPF*.

epimerase in combination with chondroitin structure in NSCLC patients is needed.

Fan et al explored the role of *CHPF* in glioma, a lentiviral vector expressing *CHPF* shRNA was constructed and transfected into glioma U251 cells, which stably downregulated the expression levels of the *CHPF* gene in U251 cells in vitro.⁸ The results confirmed that *CHPF* promotes growth and inhibits apoptosis in glioma U251 cells. In different studies, researchers have reported that the shRNA expression vector transfected with different genes controlled the propagation, invasiveness, cell cycle, and apoptosis of tumor cells.¹⁵ These observations suggest that gene knockdown strategy has the potential to be used for the treatment of cancers. Yet, no report has used this strategy to repress the expression of

CHPF gene in NSCLC. Therefore, we selected A549 cell lines to investigate the effect of *CHPF* knockdown in vitro, to explain its role in NSCLC progression. We found that lentivirus-mediated *CHPF* knockdown extensively repressed the proliferation of cancer cells and induced cell cycle arrest of NSCLC cells. We also observed that the cell growth rate and numbers in the sh*CHPF* group were drastically decreased compared to the control group. Our results showed that *CHPF* gene can be used as a target for the treatment of NSCLC.

Conclusion

In the current study, we highlighted the role of *CHPF* in NSCLC progression. This gene could be used as

a therapeutic target for curing NSCLC. But, our study also had some limitations. The main limitation is the lack of follow-up data for NSCLC patients and a poor assessment of whether CHPF could be a marker for prognosis in patients with NSCLC.

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Disclosure

The authors report no conflicts of interest in this work.

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