Long non-coding RNA, CHRF, predicts poor prognosis of lung adenocarcinoma and promotes cell proliferation and migration

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Abstract. Research has demonstrated that long non-coding RNAs (lncRNAs) are crucial factors in carcinogenesis. LncRNA, cardiac hypertrophy-related factor (CHRF), has been demonstrated to act as an oncogene in a variety of types of tumor. However, its biological function in lung adenocarcinoma remains to be elucidated. The present study aimed to examine the level of CHRF expression in lung adenocarcinoma tissues and cell lines, and to analyze the association between CHRF and clinicopathological characteristics, as well prognosis of patients with lung adenocarcinoma. Loss-of-function assays were performed to determine the biological function of CHRF. The expression of CHRF was markedly upregulated in lung adenocarcinoma tissues and cell lines. Patients exhibiting upregulated CHRF also demonstrated advanced Tumor-Node-Metastasis stage, lymph node metastasis and larger tumor size compared with those exhibiting downregulated CHRF. Results of Cox proportional hazards regression analysis suggested that highly-expressed CHRF may be regarded as an independent prognostic factor of prognosis. In addition, loss-of-function assays indicated that downregulation of CHRF suppressed cell proliferation, migration and invasion, and induced cell cycle arrest and apoptosis. Western blotting revealed that the phosphoinositide-3-kinase/Akt signaling pathway activity is reduced in lung adenocarcinoma following the knockdown of CHRF. Together, these results indicate that lncRNA, CHRF, may serve a critical role in the development and progression of lung adenocarcinoma, and may act as a novel prognostic biomarker and therapeutic target in lung adenocarcinoma.

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Introduction

Lung cancer is one of the leading causes of cancer-associated mortality, with a 5-year-survival rate of 17% worldwide (1). Non-small cell lung cancer (NSCLC) accounts for 85% all lung cancer cases in China, in 2017 (2). NSCLC can be subcategorized into adenocarcinoma (LAD), squamous cell carcinoma (SCC) or large cell carcinoma. Despite improvements in chemotherapy, radiation and surgical treatments, lung cancer remains accountable for a large proportion of cancer-associated mortality (3). The average 5-year survival rate of lung cancer is approximately <15% in the urban areas of China (4-7). The increase in incidence of LAD has caused socioeconomic developmental and environmental concerns (8), and the underlying mechanisms of LAD remain unclear.

Long non-coding RNAs (lncRNAs) are a class of RNA transcripts >200 nucleotides in length, with no clear protein-coding ability (9). Thus far, >300 lncRNAs have been annotated in the lncRNA database, the majority of which have been studied in humans (10). Previous studies have demonstrated that lncRNAs serve important roles in various biological processes in numerous diseases, including various forms of cancer (9,11-13). LncRNAs function in the regulation of complicated mechanisms and participate in physiological, pathological and cytobiological functions, including apoptosis, cell proliferation and chemoresistance (14-16). LncRNA cardiac hypertrophy-related factor (CHRF) has recently been reported to act as an oncogene; Qiuyun Wu *et al* (17) demonstrated that lncRNA CHRF functions as an endogenous 'sponge' of micro RNA (miR)-489, repressing miR-489 activity and functioning in pulmonary fibrosis.

In the present study, it was revealed that the expression of CHRF in LAD tissues and cell lines was increased compared with the negative controls. Loss-of-function assays were performed to analyze the effects of CHRF on the proliferation, cell cycle, apoptosis, migration and invasion of LAD cells. Western blotting was performed to study the relevance of CHRF in the phosphoinositide-3-kinase (PI3K)/Akt signaling pathway. These experiments indicated that CHRF may be considered as a novel prognostic factor and a therapeutic target for LAD.

Materials and methods

Tissues samples. A total of 80 LAD tissues and matched adjacent normal tissues were collected from patients

treated at the First Affiliated Hospital of Chinese PLA General Hospital (Beijing, China) between August 2010 and August 2013. The age of these patients range from 45 to 75 years (mean age: 55 years). The sex ratio of these patients is 38 males and 42 females). Inclusion criteria: Patients with lung adenocarcinoma who have never received radiotherapy or chemotherapy. Exclusion criteria: Patients who were diagnosed as pneumonia in addition to LAD or those whose course of disease was not visited and recorded. The present study was a retrospective study. The LAD diagnosis of all tissues had been histopathologically confirmed, and frozen in liquid nitrogen. All patients provided written informed consent for use of tissue in the present research. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Chinese PLA General Hospital (Beijing, China).

Cell culture and transfection. The human LAD cell lines, SPC-A1, NCI-H441 and NCI-H1975, and the normal lung epithelial cell, BEAS-2B, were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). BEAS-2B cells were incubated in complete medium [RPMI-1640+10% fetal bovine serum (FBS; Lonza Group, Ltd., Basel, Switzerland)]. LAD cells were cultured and incubated in medium (DMEM+10% FBS). All mediums were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). All cell lines were incubated in a humidified atmosphere at 37°C with 5% CO_2. The complete medium was replaced every 2-3 days.

The LAD cell lines (500 cells/well) were transfected with 50 nM small interfering RNA (si)-CHRF or si-negative control (NC) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The si-CHRF expression vector and matched scrambled control vectors were synthesized and purchased from GeneCopoeia, Inc. (Rockville, MD, USA). To obtain the optimal transfection efficiency, we used two siRNAs to knock down CHRF. They are si-CHRF#1 and si-CHRF#2. The siRNA sequences used are as follows: si-CHRF#1: 5'-TGCCTCTCTAGAGAGCAGC-3'; si-CHRF#2: (5'-CCGATCTGACATGACTGCG-3'. A total of 48 h after transfection, the cells were harvested for RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. All experiments were performed in triplicate.

RNA extraction and RT-qPCR. Total RNA was extracted from LAD tissues or cells using TRIzol (Thermo Fisher Scientific, Inc.), according to manufacturer's instructions. RNA samples were stored at -80°C. A One-Step SYBR RT-PCR kit (Takara Bio, Inc., Otsu, Japan) was used to determine the expression of CHRF using a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each reaction contained 2 μl total RNA template, 1 μl forward primer, 1 μl reverse primer and 16 μl reaction mixture. The thermocycling conditions were as follows: Stage 1 (reverse transcription reaction), 1 cycle of 42°C for 5 min, 95°C for 10 sec; Stage 2 (PCR reaction), 40 cycles of 95°C for 5 sec, 60°C for 34 sec; Stage 3 (dissociation curve analysis), 95°C for 15 sec, 40 cycles of 65°C for 1 min and 95°C for 14 sec. The primers used for CHRF and GAPDH were purchased from Sangon Biotech Co., Ltd.

(Shanghai, China). The primers are as follows: Human CHRF forward primer: 5'-AGATTCACATGGTATCCTGAAC'; reverse: 5'-TAGTCTGGCCACATTTTGTCTC-3'. GAPDH forward primer: 5'-TGTGTCCGTCGTGGATCTGA-3'; reverse: 5'-CCTGCTTCACCACCTTCTTGA-3'. All experiments were performed in triplicate. The qPCR quantification was conducted according to the $2^{-\Delta\Delta Cq}$ method (18).

Cell proliferation assay. A total of either SPCA-1 or NCI-H441 $2x10^4$ cells/well were seeded in 96-well plates. A total of 20 μl MTT solution (0.5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to each well, followed by incubation at 37°C for 4 h. The cell culture medium was then carefully aspirated, and the formazan crystals were dissolved in 0.2 ml dimethyl sulfoxide. Absorbance was measured at 490 nm on a SpectraMax M5 microplate reader. All experiments were performed in triplicate.

Flow cytometry. Quantification of apoptosis was performed using an AnnexinV-FITC Apoptosis Detection kit (Beijing Biosea Biotechnology Co., Ltd., Beijing, China) 48 h after transfection, according to the manufacturers' protocol. The results were analyzed using CellQuest software v.0.9.13 (BD, Franklin Lakes, NJ, USA). Cells in the right lower quadrant were considered to be apoptotic. For cell cycle analysis, $1x10^6$ cells were fixed. To fix cell, 0.5 ml of cold PBS was used to resuspend cells. Next, the resuspended cells were added into 1.2 ml of 99.7% absolute ethyl alcohol (The final concentration is 70%). Finally, the resuspended cells were fixed at 4°C overnight. The cells were then stained with propidium iodide (50 μg/ml) at 4°C for 30 min in the dark. The cell cycle distribution was analyzed by using FlowJo 7.6.1 (FlowJo, LLC, Ashland, Oregon, USA). All experiments were performed in triplicate.

Transwell assay. Cell invasion ability of SPCA-1 and NCI-H441 cells was assessed using Costar transwell chambers (Corning Incorporated, Corning, NY, USA) containing polycarbonate membranes (6.5 mm in diameter with a pore size of 8 μm), according to the manufacturer's protocol. The transwell membranes were each coated with 80 μl Matrigel (500 ng/μl; BD Biosciences, Franklin Lakes, NJ, USA), and incubated at 37°C for 4 h. A total of $2x10^5$ cells were added to the upper compartment of each well in 200 μl of PFHM-II Protein-Free Hybridoma Medium (Gibco: Thermo Fisher Scientific, Inc.); supernatant complete medium of cells (0.5 ml) was added to the bottom chamber. Following incubation for 24 h at 37°C, the cells which had invaded to the lower chamber were stained with hematoxylin and eosin (Thermo Fisher Scientific, Inc.). Cells were fixed with 95% ethanol for 10 min at 37°C and then stained with 19% hematoxylin for 20 min and 0.5% eosin for 3 min at 37°C., Then it was counted under a light microscope at a magnification of x1,000 and the number was counted within randomly nine field for each experiment. All experiments were performed in triplicate.

Western blot analysis. Cell proteins were isolated using 400 μl of RIPA buffer (Thermo Fisher Scientific, Inc.). Gels were scanned and quantified by densitometry using
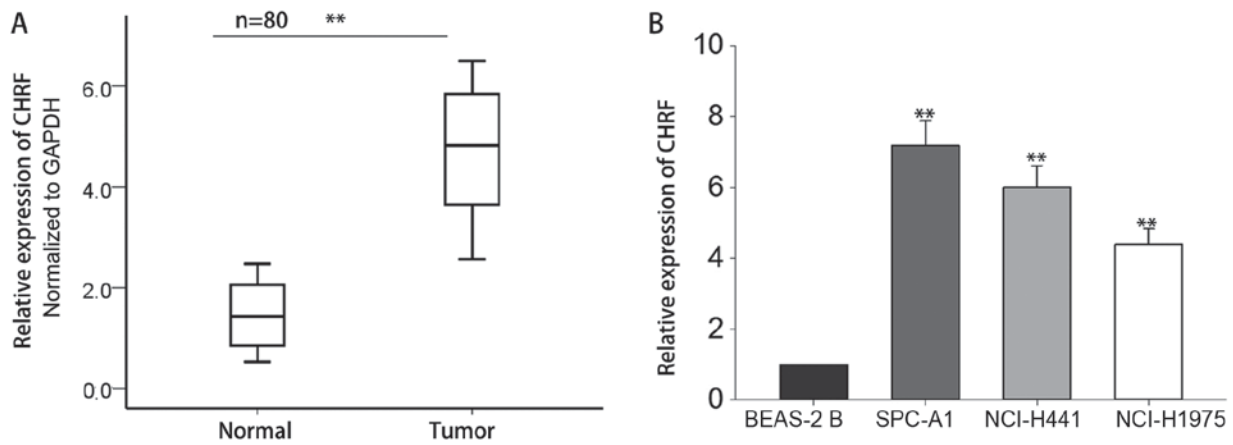
Figure 1. LncRNA CHRF expression is upregulated in lung adenocarcinoma tissues and cell lines. (A) The expression level of CHRF in LAD tissues and matched adjacent normal tissues. (B) The expression level of CHRF in LAD cell lines, SPC-A1, NCI-H441, NCI-H1975, and in normal BEAS-2B cells. Error bars represented the mean ± standard deviation of ≥3 independent experiments. **P<0.01 vs. BEAS-2 B group. LncRNA, long non-coding RNA; CHRF, cardiac hypertrophy-related factor; LAD, lung adenocarcinoma.

the Quantity-One 4.4 software (Bio-Rad, Laboratories, Inc., CA, USA). The protein was quantified by using the Bradford method (Bio-Rad, Laboratories, Inc.). The sample solution containing 50 μg proteins were separated by 10% SDS-PAGE, then transferred into nitrocellulose membranes (Merck KGaA, Darmstadt, Germany). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 1% Tween (TBS) for 1 h, then incubated with the following primary antibodies overnight at 4°C: Phosphorylated (p-)PI3K (cat no. ab125633, 1:2,000), total PI3K (cat no. ab127617, 1:2,000), p-AKT (cat no. ab38449, 1:2,000), total Akt (cat no. ab126580; 1:2,000) and GAPDH (cat no. ab8245; 1:2,000) (all from Abcam, Cambridge, UK). The secondary antibody (anti-mouse IgG) conjugated to horseradish peroxidase (cat no. 7076, 1:300; Cell Signaling Technology, Inc., Danvers, MA, USA) was incubated with the membranes for 1 h at 37°C. Protein binds were visualized using an enhanced chemiluminescence reagent chromogenic substrate (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. All experiments were performed in triplicate.

Statistical analysis. All statistical analysis was performed using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA). The data were analyzed and presented as the mean ± standard deviation. Data between two groups were analyzed by using the paired-student t-test. Multiple comparisons were made by one-way ANOVA with the Least Significant Difference post hoc test. Correlations between CHRF expression and clinicopathological features of LAD patients were analyzed by Pearson χ^2 test. Survival analysis was performed using the Kaplan-Meier method and the log-rank test was used to compare differences between patient groups. P<0.05 was considered to indicate a statistically significant difference.

Results

CHRF expression is upregulated in lung adenocarcinoma tissues and cell lines. To determine the role of the lncRNA,

CHRF, in the development and progression of lung adenocarcinoma (LAD), RT-qPCR was performed to measure the expression of CHRF in 80 pairs of LAD tissues and adjacent normal tissues. The results revealed that CHRF was significantly increased in LAD tissues compared with adjacent normal tissues (Fig. 1A, P<0.01). The expression level of CHRF was also measured in LAD cell lines and the normal lung epithelial BEAS-2B cells. As demonstrated in Fig. 1B, the level of CHRF was increased in LAD cells compared with normal epithelial cells (P<0.01). The level of CHRF was highest in SPC-A1 and NCI-H441 cells, which were selected for use in subsequent experiments. These results suggest that CHRF may serve a crucial role in the development of LAD.

Association between the expression of CHRF and clinicopathological characteristics of LAD patients. In order to explore the association between CHRF and various clinicopathological characteristics of LAD patients, the mean value of CHRF expression in LAD tissue was used as a cutoff value (patients exhibiting expression higher than the cutoff value were classified into high expression and patients exhibiting expression lower than or equal to the cutoff value were classified into low expression) and all patients were divided into a high expression group (n=41) and a low expression group (n=39). As presented in Table I, high expression of CHRF was significantly associated with advanced TNM stage, lymph node metastasis and large tumor size (P<0.05). However, there was no evident association between the expression of CHRF and other characteristics, including age, gender, smoking and differentiation (P>0.05).

Upregulation of CHRF expression is associated with poor prognosis for patients with LAD. The Kaplan-Meier and the log-rank test were used to investigate the clinical relevance of CHRF in LAD. Patients exhibiting high expression of CHRF had a markedly lower overall survival probability than those with low expression of CHRF (Fig. 2). Univariate and multivariate Cox regression analyses indicated that the expression of CHRF, tumor size and lymph metastasis were associated

Table I. Association between CHRF expression and clinico-pathological characteristics (n=80).

Variable	CHRF expression		
	Low	High	P-value
Age			
≤55	21	15	0.177
>55	18	26	
Sex			
Male	20	18	0.655
Female	19	23	
Smoking status			
Smoking	24	17	0.080
Non-smoking	15	24	
Differentiation			
Poor	17	26	0.116
Well/moderate	22	15	
Tumor size			
≤3 cm	26	13	0.003[b]
>3 cm	13	28	
TNM stage			
I-II	23	14	0.043[a]
IIIa	16	27	
Lymph metastasis			
Absent	24	14	0.025[a]
Present	15	27	

CHRF, cardiac hypertrophy-related factor; TNM, tumor-node-metastasis. Pearson χ^2 test. [a]$P<0.05$, [b]$P<0.01$.

Table II. Multivariate analysis of prognostic parameters in patients with lung adenocarcinoma by Cox regression analysis.

Variable	Multivariate	Univariate
	P-value	P-value
Age	0.944	0.940
≤60		
>60		
Sex	0.801	0.817
Male		
Female		
Smoking	0.510	0.341
Smoking		
No smoking		
Differentiation	0.907	0.872
Poor		
Well/Moderate		
Tumor size	0.001[b]	<0.001[b]
≤3 cm		
>3 cm		
TNM stage	0.105	0.111
I-II		
IIIa		
Lymph metastasis	0.048[a]	0.032[a]
Absent		
Present		
CHRF expression	0.001[b]	<0.001[b]
Low		
High		

TNM, tumor-node-metastasis; CHRF, cardiac hypertrophy-related factor [a]$P<0.05$, [b]$P<0.01$.
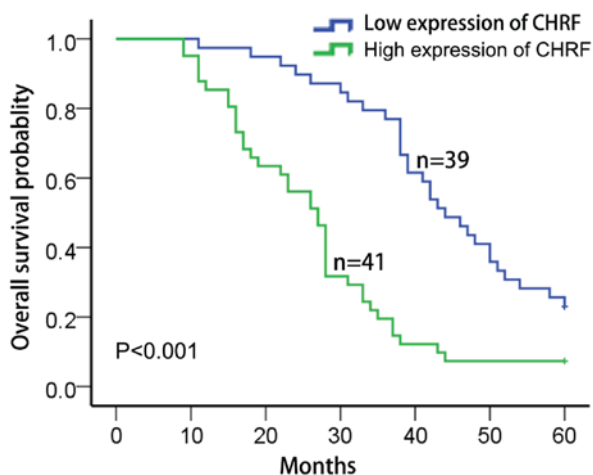

Figure 2. Kaplan-Meier analysis of the association between CHRF expression level and overall survival probability of patients with lung adenocarcinoma (P<0.001). CHRF, cardiac hypertrophy-related factor.

with the overall survival time of LAD patients. Therefore, lncRNA CHRF may act as an independent prognostic marker for the overall survival time of LAD patients (Table II).

Knockdown of CHRF represses cell proliferation by regulating cell cycle and apoptosis. Loss-of-function experiments were performed to investigate the biological function of CHRF in LAD. The results of RT-qPCR analysis revealed that the expression of CHRF was downregulated in si-SPCA-1 and si-NCI-H441 cells compared with the si-NC group (P<0.01; Fig. 3A). MTT assays demonstrated downregulation of CHRF expression significantly reduced cell proliferation both in si-SPCA-1 and si-NCI-H441 cells (P<0.01; Fig. 3B). Flow cytometry revealed that knockdown of CHRF caused cell cycle arrest most often in the G_0/G_1 phase, and induced cell apoptosis (P<0.01; Fig. 3C and D). These results indicate that knockdown of CHRF reduced cell proliferation by affecting cell cycle and apoptosis.

Inhibition of CHRF suppresses cell migration and invasion in LAD. Transwell assays were performed to further explore the effect of CHRF on the progression of LAD. This demonstrated that cells transfected with si-CHRF were less migratory compared with si-NC cells (P<0.01; Fig. 4A). Furthermore,
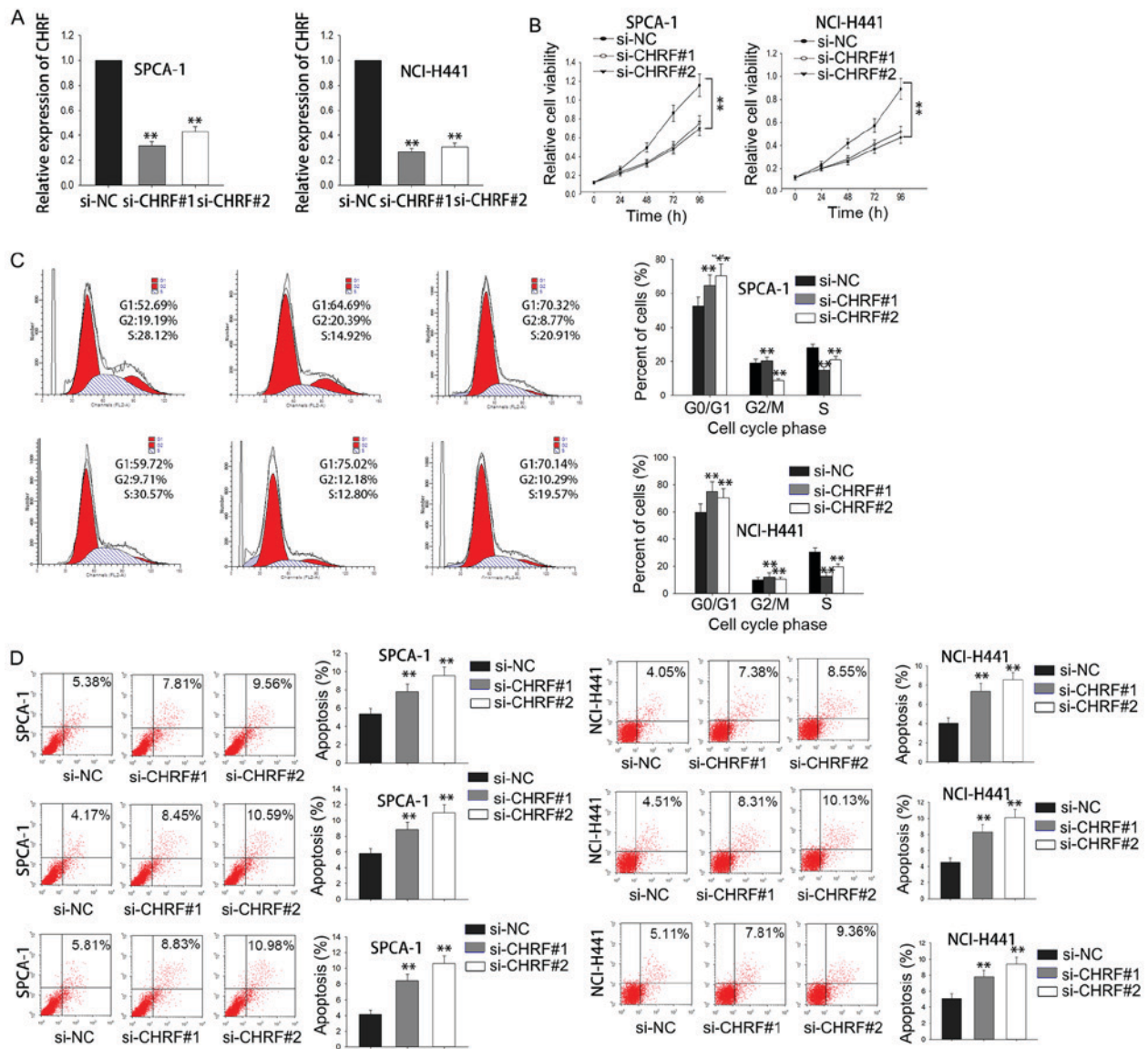
Figure 3. Knockdown of CHRF represses cell proliferation by affecting the cell cycle and apoptosis. (A) Reverse transcription-quantitative polymerase chain reaction was used to measure the expression level of CHRF in cells transfected with si-CHRF. (B) MTT assays revealed the effect of si-CHRF on cell proliferation of lung adenocarcinoma cells. (C) Flow cytometry was used to analyze the effect of si-CHRF on cell cycle. (D) Flow cytometry was used to analyze the effect of si-CHRF on apoptosis. Error bars represent the mean ± standard deviation of at ≥3 independent experiments. **P<0.01 vs. control group. CHRF, cardiac hypertrophy-related factor; si, small interfering RNA; NC, negative control.

the transwell invasion assay demonstrated that knockdown of CHRF significantly reduced the invasive ability of LAD cells (P<0.01; Fig. 4B). These data suggest that CHRF functioned as an oncogene in the development and progression of LAD.

Knockdown of CHRF reduces the expression level of protein members of the PI3K/Akt signaling pathway in LAD. To explore the potential mechanism through which CHRF affects the progression of LAD, western blotting was performed to determine the effect of downregulated CHRF on the PI3K/Akt signaling, which has been demonstrated to be ectopically activated in human cancers, increasing cell proliferation and metastasis (19). Western blotting revealed that knockdown of CHRF dramatically reduced the protein level of p-PI3K and p-Akt in LAD (P<0.01; Fig. 5). These data indicate that the PI3K/Akt signaling pathway may

function in the proliferation and metastasis of LAD cells induced by CHRF.

Discussion

Dysregulated expression of lncRNAs has been demonstrated to contribute to the development and progression of various types of human cancer, providing novel therapeutic targets for cancer treatment and drug resistance (20-22). LncRNA CHRF has been reported to function in numerous human diseases, including a variety of forms of cancer. LncRNA CHRF-induced downregulation of miR-489l promotes metastasis of colorectal cancer via twist family BHLH transcription factor 1/epithelial-mesenchymal transition signaling pathway (23). Previous studies have reported that miR-489 is regulated by lncRNA CHRF (17,24). CHRF also
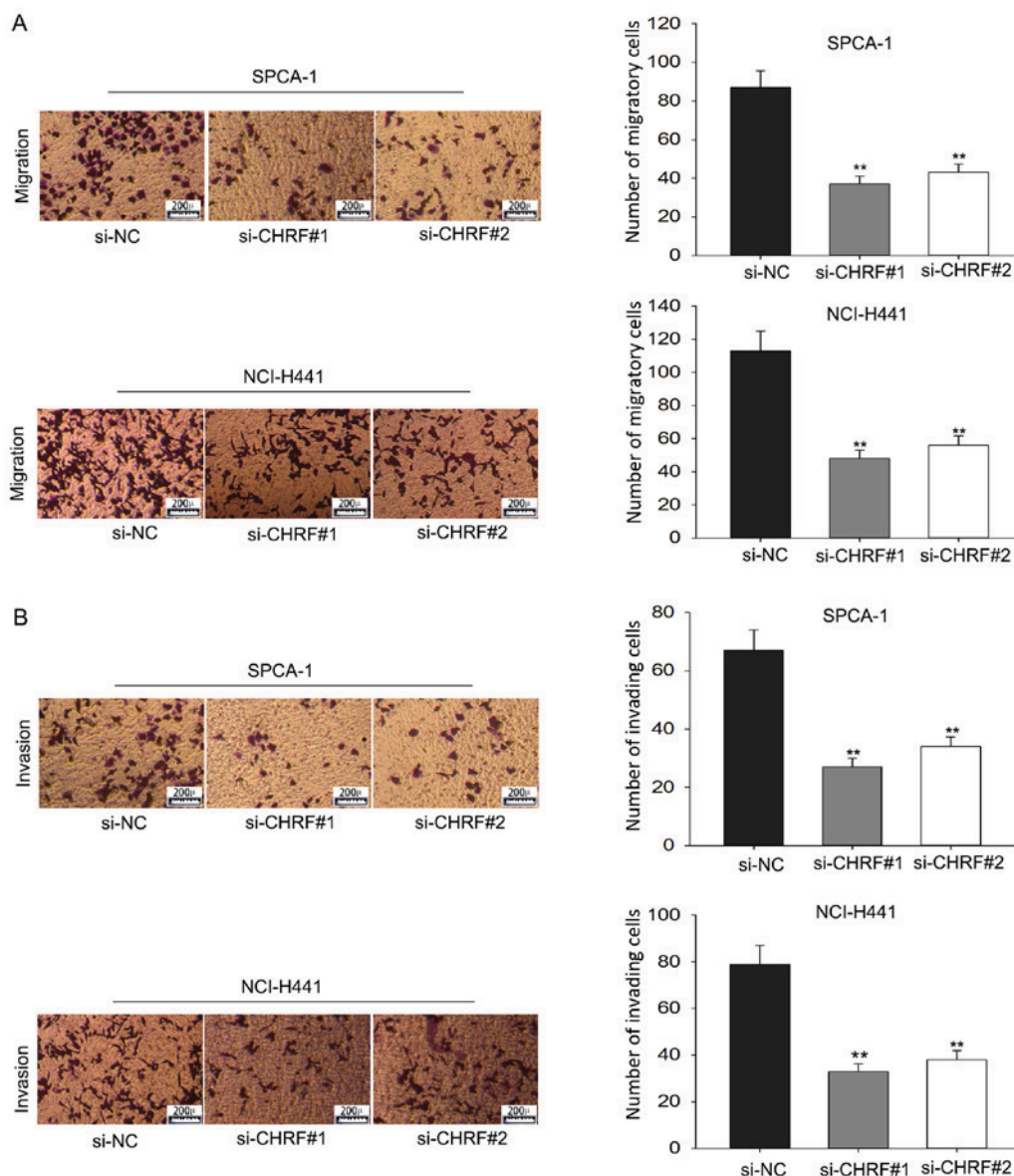
Figure 4. Knockdown of CHRF suppresses cell migration and invasion abilities of LAD cells. (A) Transwell assays demonstrated that knockdown of CHRF inhibited the migratory abilities of LAD cells. (B) Quantification of the transwell assay results showed that the invasive ability of LAD cells was suppressed by CHRF knockdown. n=200 μm. Error bars represented the mean ± standard deviation of ≥3 independent experiments. **$P<0.01$ vs. control group. CHRF, cardiac hypertrophy-related factor; LAD, lung adenocarcinoma; SI, small interfering RNA; NC, negative control.

serves critical roles in leukemia (25), human erythroleukemia (26) and myeloid leukemia (27), and is a key regulator the pathology of heart failure (28). However, the molecular mechanisms underlying the process of LAD tumorigenesis remain insufficiently characterized.

The present study demonstrated that CHRF was dramatically overexpressed in LAD tissues and cell lines compared with normal tissues and cells. Overexpression of CHRF was associated with advanced TNM stage, lymph node metastasis and large tumor size. It was also revealed that patients exhibiting high expression of CHRF had a shorter overall survival time compared with those exhibiting low expression of CHRF. Furthermore, loss-of-function assays indicated that knockdown of CHRF inhibited cell proliferation, migration and invasion of LAD.

The PI3K/Akt pathway is a crucial intracellular signaling pathway involved in proliferation and EMT in the various types of cancer (29). It has been demonstrated that lncRNAs serve important roles in PI3K/Akt signaling pathway. For example, it has been reported that downregulation of metastasis associated lung adenocarcinoma transcript 1 induced EMT via the PI3K/Akt pathway in breast cancer (30). Downregulation of lncRNA MALAT1 induces epithelial-to-mesenchymal transition via the PI3K-AKT pathway in breast cancer (31). In the present study, it was demonstrated that knockdown of CHRF decreased the protein expression levels of p-PI3K and p-Akt, suggesting that the PI3K/Akt pathway may function in the CHRF-induced carcinogenesis of LAD.

Overall the present study indicates that CHRF expression is enhanced during the progression of LAD. Overexpression of CHRF was associated with advanced TNM stage, lymph node metastasis and large tumor size in patients with LAD. Downregulation of CHRF expression reduced cell proliferation and metastasis in LAD. Furthermore, knockdown of
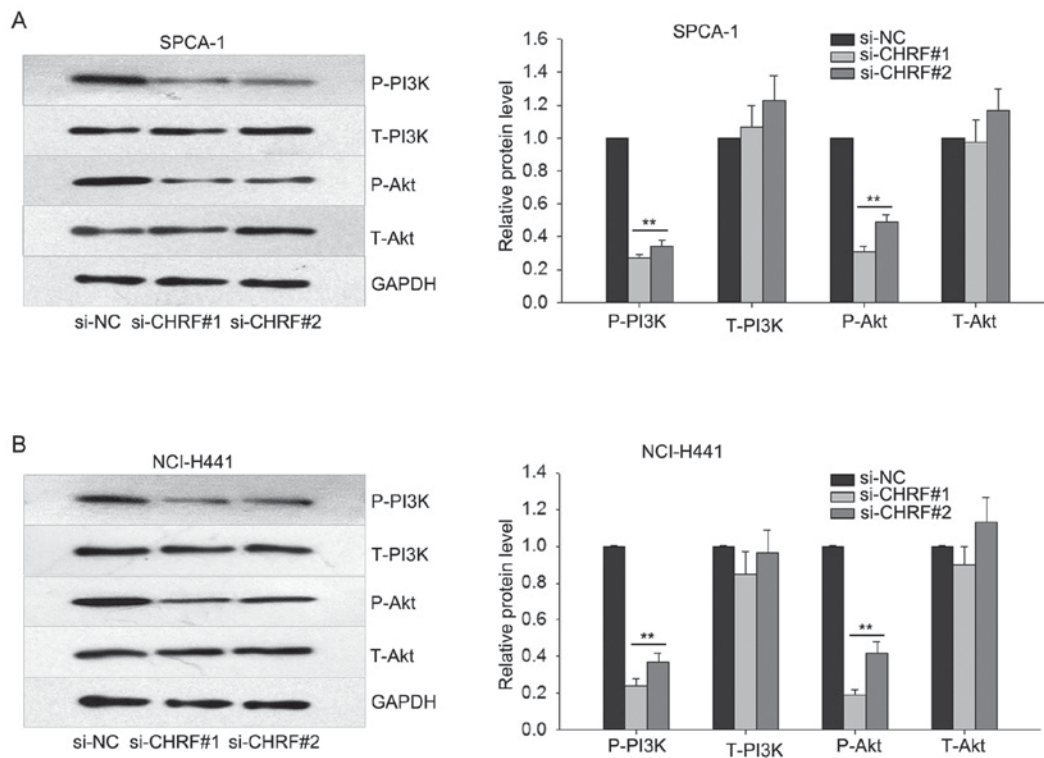
Figure 5. Knockdown of CHRF reduces the protein expression level of PI3K/Akt pathway in LAD. Western blotting revealed that knockdown of CHRF decreased the protein expression levels of p-PI3K and p-Akt in (A) SPCA-1 and (B) NCI-H441 cells. Error bars represented the mean ± standard deviation of ≥3 independent experiments. **$P<0.01$ vs. control group. CHRF, cardiac hypertrophy-related factor; PI3K, posphoinositide-3-kinase; LAD, lung adenocarcinoma; p-, phosphorylated; T-total; si, small interfering RNA; NC, negative control.

CHRF resulted in reduced activity of the PI3K/Akt signaling pathway. Thus, CHRF may be a novel molecular target for the diagnosis and therapy of LAD.

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Availability of data and materials

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

Authors' contributions

XXie, WZ, JP, XXio, HW and LM were responsible for the completion of experiments. XXie and WZ undertook study design and wrote the manuscript.

Ethics approval and consent to participate

All patients provided written informed consent for the use of their tissue in the present research. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Chinese PLA General Hospital (Beijing, China).

Consent for publication

All patients, researchers and authors participated in this study have provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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