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Cyclophilin B promotes cell proliferation, migration, invasion and angiogenesis via regulating the STAT3 pathway in non-small cell lung cancer

Mao-rong Teng[a,b], Jian-an Huang[a,*], Zheng-tai Zhu[b], Hua Li[b], Jiang-feng Shen[c], Quan Chen[c]

[a] Department of Respiratory, The First Affiliated Hospital of Soochow University, Soochow, 215006, China
[b] Department of Geriatrics, Taizhou People's Hospital, TaiZhou, 225300, China
[c] Department of Cardiothoracic Surgery, Taizhou People's Hospital, TaiZhou, 225300, China

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ABSTRACT

Lung cancer is the most common type of cancer and has become the leading cause of cancer-associated mortality worldwide. It has been reported that expression of Cyclophilin B was greatly elevated in the pancreatic cancer patient sera as compared with the healthy volunteer sera. This study aimed to investigate the role and regulatory mechanism of CypB in NSCLC progression. The expression levels of CypB was detected in NSCLC samples and cell lines by ELISA, western blot and immunohistochemistry assay. In addition, CCK8, colony formation, scratch and transwell assays were used to evaluate the proliferation, migration and invasion of A549 cells with CypB silencing. The expression of angiogenesis related proteins and pathway-related factors were detected by western blot. In NSCLC samples, CypB expression was upregulated. The expression of CypB was significantly reduced in the siRNA-cyclophilin B group. In addition, CypB silencing inhibited cell proliferation, migration and invasion. The expression of angiogenesis related proteins and pathway-related factors have also changed significantly. These findings suggested that CypB silencing may suppress the proliferation, invasion, migration and angiogenesis of A549 cells via inhibiting STAT3 pathway.

1. Introduction

Lung cancer is the most common type of cancer and has become the leading cause of cancer-associated mortality worldwide, which has a low 5-year survival rate of 16.6% [1–3]. Non-small cell lung cancer (NSCLC) covers around 85% of all lung cancers, including specific subtypes such as adenocarcinoma and squamous cell carcinoma [4,5]. Although there has been great progress in chemotherapy, surgical and targeting therapy for NSCLC patients in last few decades, the 5-year survival rate is still extremely low [6,7]. Therefore, a better understanding of detailed molecular mechanisms in NSCLC progression is critically important for advancing the diagnosis and personalized therapy.

Cyclophilin B (CypB) is an ER-resident protein with peptidyl-prolyl cis/trans-isomerase activity [8,9]. Previous study suggested that CypB is mainly expressed in the endoplasmic reticulum and involves in multiple functions, including immunosuppression [10], hepatitis virus replication [11] and prolactin signaling [12]. In addition, study research has shown that expression of CypB was greatly elevated in the pancreatic cancer patient sera as compared with the healthy volunteer sera [13]. It has been reported that CypB is involved in Stat3 activation and in generation of reactive oxygen species in other cancer cells [14,15]. However, the biological role and regulatory mechanism of CypB in NSCLC progression remains largely unclear.

The present study aimed to investigate the CypB expression in NSCLC tissues and cell lines. The finding revealed that CypB was overexpressed in NSCLC and inhibition of CypB may effectively impede cell proliferation, migration, invasion and angiogenesis via regulating the STAT3 pathway. These results may provide a potential target for NSCLC treatment.

2. Materials and methods

2.1. Clinical samples

A total of 30 pairs of NSCLC tissues, paracarcinoma tissues and fresh

* Corresponding author at: Department of Respiratory, The First Affiliated Hospital of Soochow University, No. 899, Ping Hai Road, Gusu District, Soochow, Jiangsu, 215006, China.
E-mail address: Huang_jian_an@163.com (J.-a. Huang).

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blood sample were collected from the First Affiliated Hospital of Soochow University (Soochow, China), and no patients had undergone radiotherapy and chemotherapy (age range, 65–85 years old). All the collected samples were frozen liquid nitrogen and stored at −80 °C until use. This study was approved by the ethics committee of the First Affiliated Hospital of Soochow University and all patients provided written informed consent.

2.2. Cell culture and transfection

Human NSCLC cell lines including A549, H441, H1975 and the normal human bronchial epithelial (HBE) cell line were obtained from ATCC (Manassas, VA, USA). All cells were cultured in the DMEM medium (Gibco, MA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin G and 100 μg/ml streptomycin (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an atmosphere of 5% CO_2 at 37 °C.

In the present study, siRNA-cyclophilin B-1 and siRNA-cyclophilin B-2 were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Negative control siRNA was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). siRNA-cyclophilin B was transfected into A549 cells using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Subsequently, the transfected cells were incubated for 72 h. The experimental groups were as follows: control group, siRNA-NC group, siRNA-cyclophilin B-1 group and siRNA-cyclophilin B-2 group. Then, A549 cells were treated with the agonist of JAK2/STAT3 (IL-11, 10 ng/mL) for 24 h. The experimental groups were as follows: control group, siRNA-NC group, siRNA-cyclophilin B group and siRNA-cyclophilin B + IL-11 group.

2.3. Quantitative real time PCR (qRT-PCR)

TRIzol® reagent (Thermo Fisher Scientific, Inc.) was applied for RNA extraction. Total RNA was reverse transcribed into cDNA using an RT kit (Takara Biotechnology Co., Ltd., Dalian, China) following the manufacturer's instructions. qPCR was performed using the SYBR Premix Ex Taq (Applied Biosystems; Thermo Fisher Scientific, Inc.) with cycling conditions of 95 °C for an initial 10 min followed by 40 cycles of denaturation for 30 s at 95 °C, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. GAPDH was used to normalize cyclophilin B expression. The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method. The following primers were used for amplification: GAPDH, forward 5′-ATTGATGGATGCTAFGAGTATT-3′, reverse 5′-AGTCTTCTG GGTGGCAGTGAT-3′; cyclophilin B, forward 5′-AATTCCATCGTGTAAT CAAGGACTT-3′, reverse 5′-TCTTGACTGTCGTGATGAAGAACT-3′.

2.4. ELISA assay

After blood samples were collected and blood serum was extracted by centrifugation at 15,000 ×*g* at 4 °C for 10 min. The expression level of cyclophilin B in plasma was determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (BD Pharmingen; BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocols.

2.5. Cell viability assay

Cell viability was detected by Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, MD, Japan). The cells harvested after 24 h of transfection were seeded into 96 well plates. Then the cells were then incubated at 37 °C with 5% CO_2 for 12, 24, and 48 h. Furtherly, 10 μl CCK-8 solution was added into each well and the cells were incubated at 37 °C for 2 h. Finally, the optical density (OD) was read at 490 nm using a Bio-Rad iMark plate reader.

2.6. Colony formation assay

The colony formation assay was performed after 48 h transfection. Cells (500 cells/well) were seeded into 6-well plates and incubated for 10 days in 5% CO_2 at 37 °C to form colonies. Cells were fixed in methanol for 15 min at 4 °C and stained with 1% crystal violet solution for 30 min at room temperature. The colonies were observed and images were captured under a light microscope.

2.7. Scratch assay

Cells were collected and seeded into a 6-well plate at density of 5×10 [5] cells/well, and then cultured to reach 100% confluence. Across the center of the well, the cell monolayer was scratched with a 10 μL pipette tip. Subsequently, these cells were cultured for 24 h. An inverted microscope (CKX41; Olympus Corporation, Tokyo, Japan) was used for observation and photography, and images of the migrated cells were analyzed using Image J v1.8.0.

2.8. Transwell assay

Transwell culture inserts (poresize, 8 mm; Falcon; BD Biosciences, Franklin Lakes, NJ, USA) were placed into the wells of 6-well plates and coated with a layer of Matrigel (BD Biosciences). Transwell plates were incubated for 1 h in a 37 °C incubator containing 5% CO_2 for gel formation. FBS was used to hydrate the membrane 2 h prior to use. Subsequently, Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.; 600 μl) containing 10% FBS was added to the lower chamber, whereas 1×10^5 cells/well were added to the upper chamber. After 72 h at 37 °C, the numbers of invaded cells on the lower chamber were calculated using a counting chamber under an inverted microscope (CKX41; Olympus Corporation).

2.9. Immunohistochemistry (IHC) assay

Formalin-fixed and paraffin-embedded samples were cut into 5 μm thick sections. Then, routine deparaffinization and rehydration were performed. The slides were incubated with 3% H_2O_2 to eliminate endogenous peroxidase, and blocked with 5% goat serum. Sections were incubated with specific antibody against cyclophilin B (1:1000; cat. no. 43603; Cell Signaling Technology, Inc.) for 1 h at room temperature, followed by the incubation of secondary antibodies (#7074, 1:2,000; Cell Signaling Technology, Inc.) for 60 min. Afterwards, the sections were treatment with DAB for 5 min. Images of staining were captured under an inverted microscope (CKX41; Olympus Corporation) and were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

2.10. Western blotting

The protein concentration of the fresh NSCLC tissue samples and cultured cells were evaluated by the BCA assay kit (Beyotime Institute of Biotechnology) following the manufacturer's instructions. Equal amounts of protein were separated by SDS-PAGE on 10% gels and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked with 5% nonfat milk for 1 h at room temperature and then probed with the following primary antibody: anti-CypB (1:1000; cat. no. 43603), anti-VEGF (1:1000; cat. no. 9698), anti-Ang2 (1:1000; cat. no. 79299), anti-phospho (p)-JAK2 (1:1000; cat. no. 3776), anti-p-STAT3 (1:1000; cat. no. 9145), anti-total (t)-JAK2 (1:2000; cat. no. 3230), anti-t-STAT3 (1:2000; cat. no. 12640) and anti-GAPDH (1:2,000; cat. no. 5174) from Cell Signaling Technology, Inc. (Danvers, MA, USA) at 4 °C overnight. The membranes were then incubated in the HRP-conjugated secondary antibody for 2 h at room temperature. Relative quantification of protein expression was analyzed with Image-Pro Plus software (version 6.0)

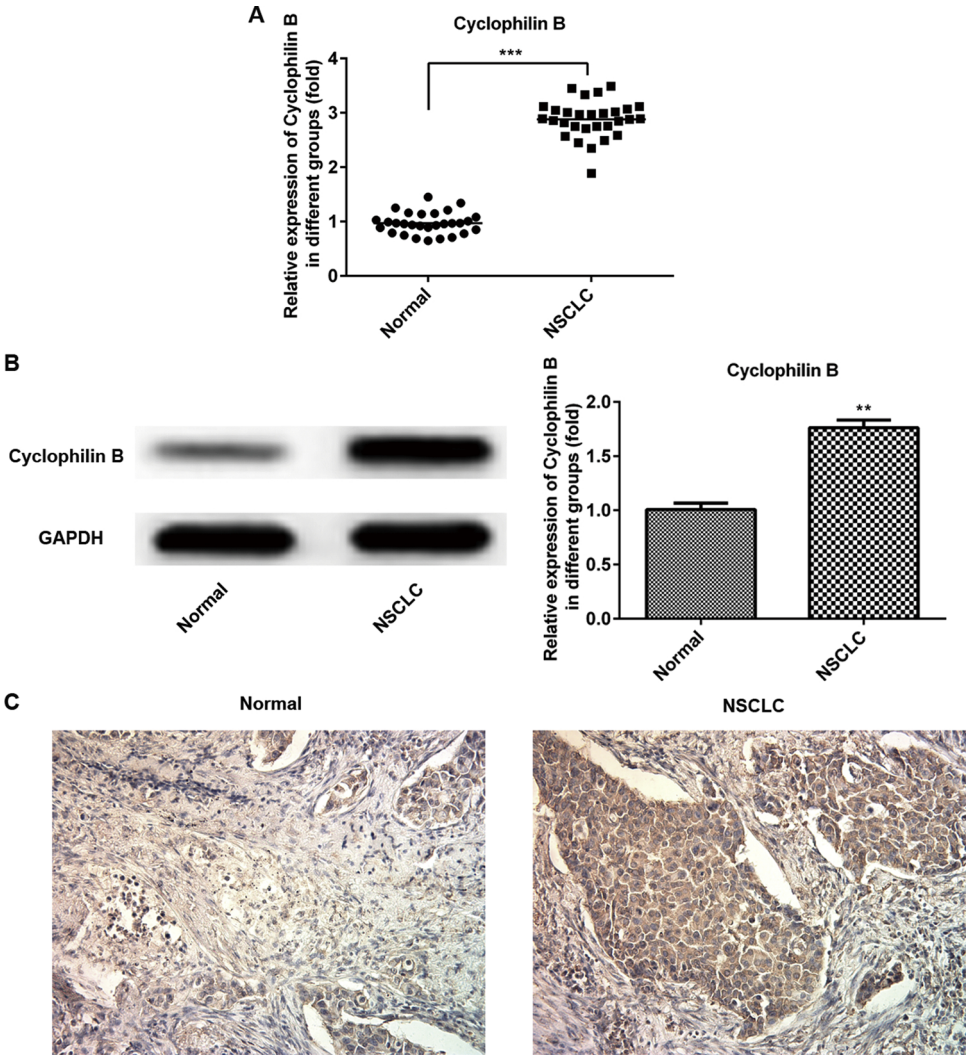

Fig. 1. Expression of CypB in NSCLC samples. The expression level of CypB were determined by ELISA (A), western blot (B) and immunohistochemistry assay (C). **P < 0.01, ***P < 0.01 vs. Normal group. CypB, Cyclophilin B; ELISA, enzyme-linked immunosorbent assay; NSCLC, non-small cell lung cancer.

based on the Integrated Optical Density (IOD) of the blotting bands.

2.11. Statistical analysis

Statistical analysis was carried out using SPSS version 20.0 software (IBM Corp, Armonk, NY, USA). All data were presented as the mean ± standard deviation. Variance ANOVA followed by the Dunnett's post hoc test was used for multiple group comparisons. The threshold for statistical significance were $P < 0.05$.

3. Results

3.1. Cyclophilin B is upregulated in NSCLC tissues and cell lines

The expression level of Cyclophilin B in plasma of 30 patients was examined using ELISA. As shown in Fig. 1A, Cyclophilin B was significantly highly expressed in NSCLC group compared to normal samples. In addition, the Cyclophilin B expression levels were measured in 30 NSCLC tissue samples by performing western blot and Immunohistochemistry assay. As shown in Fig. 1B and C, Cyclophilin B expression was significantly increased in NSCLC tissues compared to adjacent normal samples. Higher mRNA and protein expression of Cyclophilin B were also observed in NSCLC cell lines, including H441, H1975 and A549 (Fig. 2A and B). The A549 cell line exhibited the highest Cyclophilin B expression, thus, was selected for subsequent experimentation.

3.2. Cyclophilin B silencing suppresses NSCLC cell proliferation and colony formation

The interference efficiency of Cyclophilin B siRNA was determined via qRT-PCR and western blot. As shown in Fig. 2C and D, the expression of Cyclophilin B was significantly reduced in the siRNA-cyclophilin B-1 and siRNA-cyclophilin B-2 groups compared with the siRNA-NC group. The siRNA-cyclophilin B-1 plasmid, which exhibited the greatest interference effect, was selected for subsequent experimentation. Then, CCK-8 assay showed that Cyclophilin B silencing significantly inhibits the proliferation of A549 (Fig. 3A). Similarly, the capacity to form colonies was significantly decreased compared with the siRNA-NC group (Fig. 3B and C).

3.3. Cyclophilin B silencing inhibits the migration and invasion of A549 cells

The migration and invasion of transfected A549 cells were determined by scratch and transwell assays, respectively. As shown in Fig. 4A–D, cell migration and invasion were significantly inhibited in the siRNA-cyclophilin B group compared with siRNA-NC group.

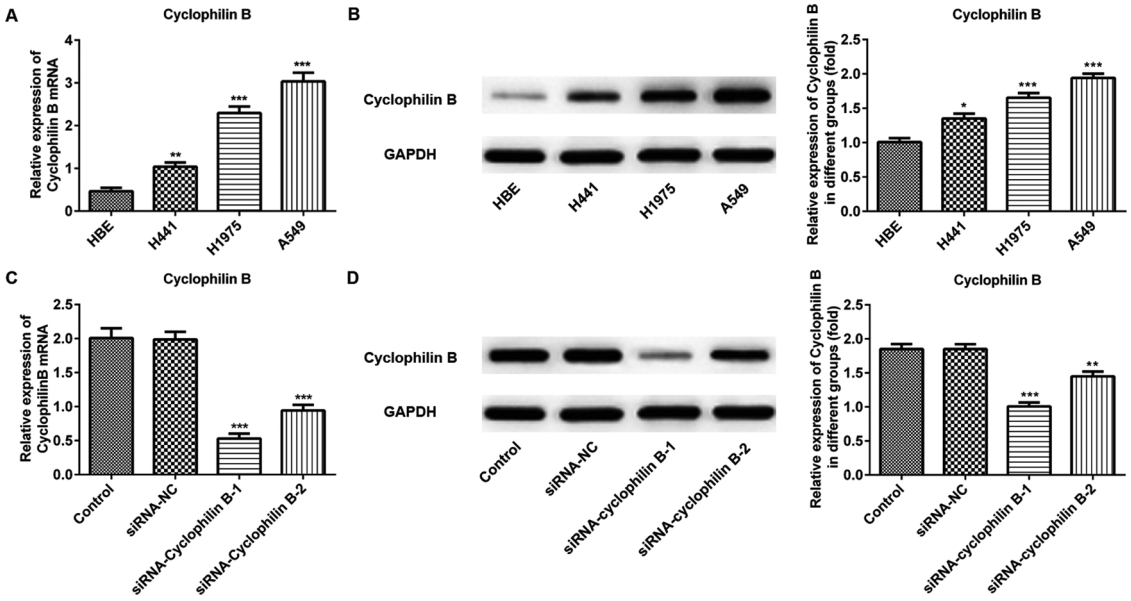

Fig. 2. Upregulation of CypB in various non-small cell lung cancer cell lines. (A, B) The expression levels of CypB in the normal human bronchial epithelial (HBE) cell line and the non-small cell lung cancer cell lines were determined by qRT-PCR and western blot. *P < 0.05, **P < 0.01, ***P < 0.001 vs. HBE cells. (C, D) Interference efficiency was also measured via qRT-PCR and western blot. **P < 0.01, ***P < 0.001 vs. control group and siRNA-NC group. CypB, Cyclophilin B; siRNA, small interfering RNA; NC, negative control.

Therefore, Cyclophilin B silencing might inhibit cell migration and invasion of A549 cells.

3.4. Cyclophilin B silencing inhibits the expression of angiogenesis related proteins

Angiogenesis is a hallmark of cancer, and a large number of studies have demonstrated that angiogenesis plays an important role in facilitating metastasis. To explore whether Cyclophilin B had an effect on angiogenesis, the expression levels of angiogenesis related proteins were detected by western blot. As shown in Fig. 5A, compared with the siRNA-NC group, the expression of angiogenesis related proteins VEGF and Ang2 were markedly decreased in siRNA-cyclophilin B group. The results demonstrate that Cyclophilin B silencing inhibit angiogenesis.

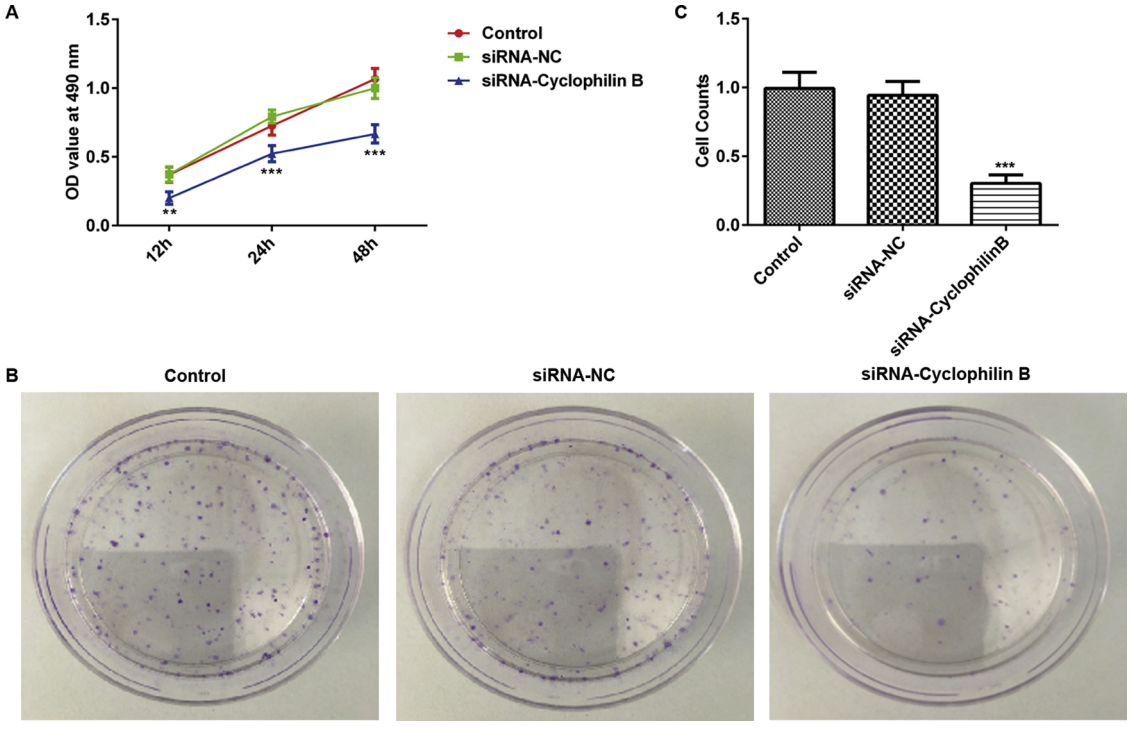

Fig. 3. Cyclophilin B silencing suppresses NSCLC cell proliferation and colony formation. (A) CCK8 and (B) colony formation assays were performed to measure cell proliferation. (C) Colony formation assay was quantitatively analyzed. **P < 0.01, ***P < 0.001 vs. control group and siRNA-NC group. CypB, Cyclophilin B; siRNA, small interfering RNA; NC, negative control.

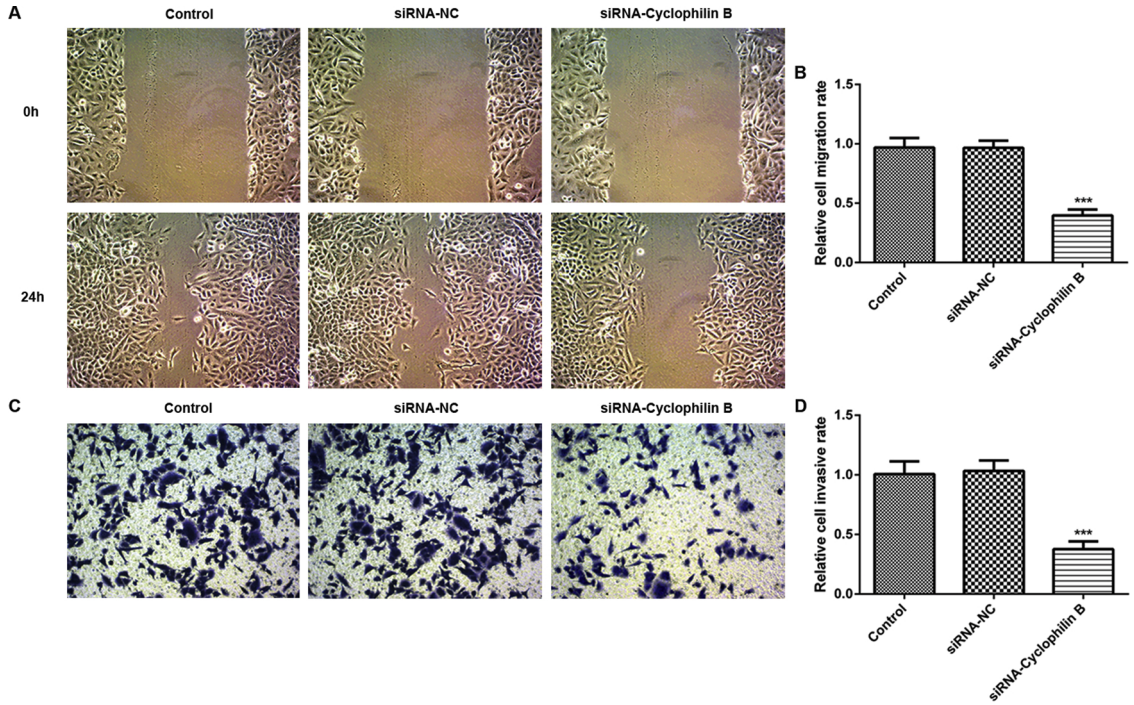

Fig. 4. Cyclophilin B silencing inhibits the migration and invasion of A549 cells. (A, B) Migration of A549 cells was detected by scratch assay. (C, D) Invasion of A549 cells was detected by transwell assay. Magnification, ×200. ***P < 0.001 vs. control group and siRNA-NC group. CypB, Cyclophilin B; siRNA, small interfering RNA; NC, negative control.

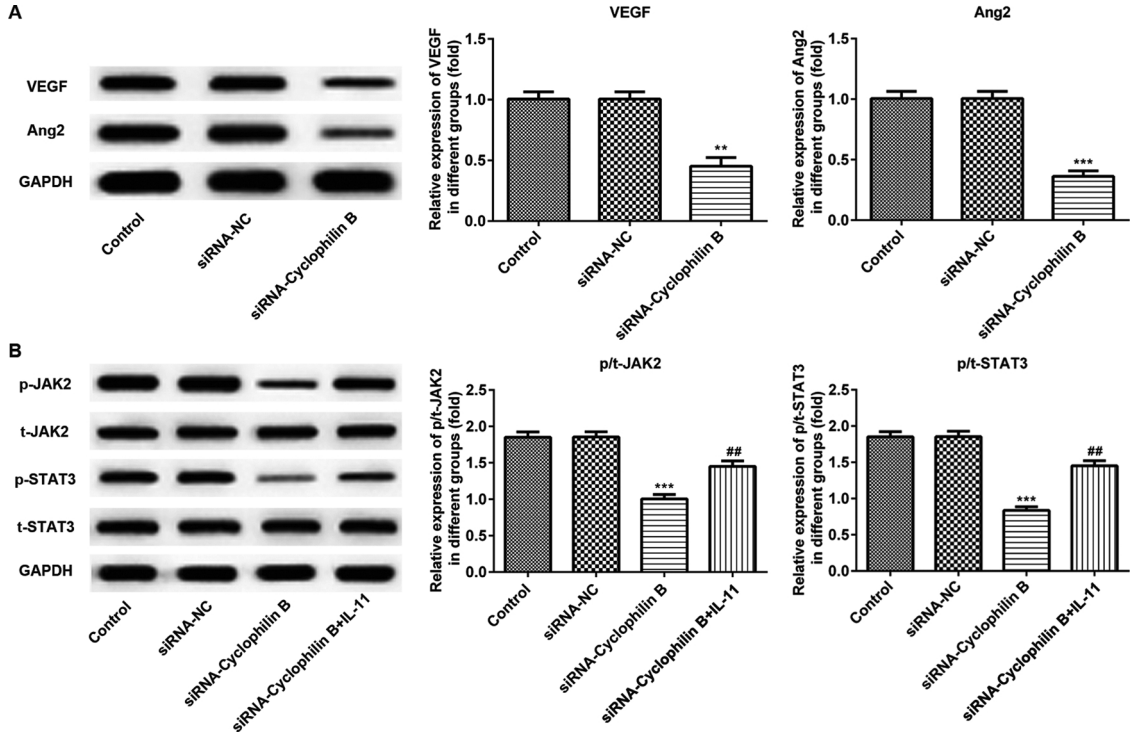

Fig. 5. Cyclophilin B silencing suppresses the proliferation, invasion, migration and angiogenesis of A549 cells via inhibiting STAT3 pathway. Western blot assay was used to detect the expression of angiogenesis related proteins (A) and the JAK2/STAT3 pathway-related factors (B). **P < 0.01, ***P < 0.001 vs. control group and siRNA-NC group. ##P < 0.001 vs. siRNA-cyclophilin B group. CypB, Cyclophilin B; siRNA, small interfering RNA; NC, negative control; IL-11, a JAK2/STAT3 agonist; p-JAK2, phosphorylation JAK2; t-JAK2, total JAK2; p-STAT3, phosphorylation STAT3; t-STAT3, total STAT3.

3.5. Cyclophilin B silencing suppresses the proliferation, invasion, migration and angiogenesis of A549 cells via inhibiting STAT3 pathway

To verify whether Cyclophilin B exerts its biological function through the STAT3 pathway, western blot assay was used to detect the expression of pathway-related factors. As shown in Fig. 5B, compared with the siRNA-NC group, the phosphorylation levels of JAK2 and STAT3 was visibly decreased in siRNA-cyclophilin B group, while the

expression of total (t)-JAK2 and t-STAT3 remained unchanged. Moreover, compared with the siRNA-cyclophilin B group, the phosphorylation levels of JAK2 and STAT3 was increased in the siRNA-cyclophilin B + IL-11 group. These results suggested that Cyclophilin B silencing inhibited STAT3 signaling pathway.

4. Discussion

In recent, Cyclophilin B has been shown to be highly expressed in breast, liver, colon, and pancreatic cancer and to play an important role in the malignant progression of tumors [16]. Previous study showed that CypB up-regulation inhibits hypoxia-induced apoptosis, which stimulates tumor growth [17]. In addition, study has shown that expression of CypB was greatly elevated in the pancreatic cancer patient sera as compared with the healthy volunteer sera [13]. The present study demonstrated that CypB was upregulated in NSCLC samples and cell lines. Subsequently, CypB silencing resulted in the inhibition of proliferation, migration and invasion as well as colony-formation viability of NSCLC cells. The same cancer-promoting effect was also found in gastric cancer, downregulation of CypB significantly inhibited growth and proliferation of gastric cancer cells, cell cycle progression, and in vivo tumorigenicity capacity [18]. Meanwhile, VEGF is an essential biological marker to evaluate angiogenesis and lymphangiogenesis [19], which has been shown to play an important role in the angiogenesis of breast cancer and closely associated with breast cancer survival [20]. The VEGF receptor (VEGFR) is closely associated with such biological behaviors, such as the occurrence, infiltration, endothelial cell proliferation, metastasis, etc., of tumor vessels [21]. A previous study reported that the VEGF family induce angiogenesis in general in oral cancer [22,23] and is associated with lymph node metastasis in gastric cancer [18]. So far, many angiogenic factors, such as VEGF and Ang2 have been identified and shown to be produced by a variety of different tumor cells, which may be play a role for survival for NSCLC patients [24]. In the present study, the expression levels of angiogenesis related proteins, VEGF and Ang2, were markedly decreased in NSCLC cells after treatment with siRNA-cyclophilin B. These results indicates that the interference of cyclophilin B may play a potential role of antiangiogenic therapy in suppressing the development of NSCLC.

Signal transducer and activator of transcription 3 (STAT3) is a critical transcription factor [25,26] that regulates cellular apoptosis, proliferation, and metastasis by the modulation of varied gene expression [27]. It has been found to be persistently activated in diverse malignancies [28]. The present study showed that CypB silencing inhibited the phosphorylation levels of JAK2 and STAT3 in NSCLC cells, which is in concordance with the previous findings. The result above shows that the promoting cancer role of CypB in NSCLC may be achieved by modulating JAK2/STAT3 pathway.

In conclusion, the results from the present study indicate that CypB silencing suppressed cell proliferation, migration, invasion and angiogenesis via regulating the STAT3 pathway in non-small cell lung cancer, suggesting that CypB has the potential to become a therapeutic target for the treatment of non-small cell lung cancer.

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No.

Availability of data and materials

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

Authors' contribution

JA H designed the current study. MR T, ZT Z, H L and JF S performed the experiments. JA H and MR T analyzed the data. JA H drafted the manuscript. MR T and Q C interpreted data and revised the final manuscript. MR T wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental protocols were performed in accordance with the principles of the Declaration of Helsinki and were approved by the Clinical Research Ethics Committee of the First Affiliated Hospital of Soochow University (Soochow, China).

Competing interests

The authors declare that they have no competing interests.

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