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MiR-520b inhibited metastasis and proliferation of non-small cell lung cancer by targeting CHAF1A

Y. CAI[1], Z.-Y. DONG[2], J.-Y. WANG[3]

[1]Department of Radiation Oncology, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China
[2]Department of Traditional Chinese Medicine, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China
[3]Department of Oncology, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China

Yong Cai and *Zhiyi Dong* contributed equally to this work

Abstract. – OBJECTIVE: To investigate the potential effect of miR-520b on the development of non-small cell lung cancer and to explore the underlying mechanism.

PATIENTS AND METHODS: The expression levels of miR-520b in non-small cell lung cancer (NSCLC) tissues and cells (A549), as well as corresponding adjacent normal tissues and normal human lung epithelial cells (BEAS-2B), were detected by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), respectively. Luciferase reported gene assay was performed to evaluate the interaction between miR-520b and chromatin assembly factor 1 subunit A (CHAF1A). Meanwhile, the effect of the miR-520b/CHAF1A axis on A549 cells was determined by subsequent experiments, including CHAF1A expression detection, cell proliferation, migration and invasion.

RESULTS: MiR-520b was lowly expressed in NSCLC tissues than that of corresponding adjacent normal tissues. Same results were obtained at the cellular level. To investigate the potential targets of miR-520b, we searched three publicly available algorithms, including TargetScan, miRDB and microRNA. Results indicated that CHAF1A was a direct target of miR-520b. Meanwhile, the luciferase reporter gene assay confirmed our hypothesis. Subsequent experiments demonstrated that decreased expression of CHAF1A resulting from the up-regulation of miR-520b could decelerate the proliferation, invasion and migration of lung cancer cells.

CONCLUSIONS: We discovered the inhibitory function of miR-520b in NSCLC by targeting CHAF1A. Moreover, our study revealed that the miR-520b/CHAF1A axis might be a potential therapeutic target for the treatment of NSCLC.

Key Words:
MiR-520b, Non-small cell lung cancer (NSCLC), CHAF1A.

Introduction

Lung cancer is one of the most common malignant tumors with highest morbidity and mortality over the world. The latest epidemiological study shows that the incidence of lung cancer in China ranks first among all malignant tumors. Although lung cancer has been studied more deeply in the past few decades, no significant improvement has been achieved in the early diagnosis and long-term survival rate of these patients. Most lung cancer patients are already in advanced stage when treated, therefore, neither treatment effect nor prognosis is ideal. At present, there is still a lack of effective method for early diagnosis and treatment of lung cancer. The overall survival rate of lung cancer patients is only 20%, and the 5-year survival rate is even less than 15%[1]. Traditional treatment of lung cancer cannot completely and accurately monitor its development and progression, so the treatment and prognosis of lung cancer are not ideal. The emergence of micro ribonucleic acid (miRNA) family members in the gene regulatory network provides clues and breakthrough for the research and treatment of modern non-small cell lung cancer (NSCLC)[2,3]. MiRNA is a class of endogenous, highly-conserved, non-coding, single-stranded and small-molecule RNA with about 21-23 nucleotides in length. It is formed after the single-stranded RNA precursor with hairpin structure and about 70-90 nucleotides in size, which can be processed by Dicer. MiRNAs can bind to the 3'-untranslated region of target messenger RNAs (mRNAs), eventually limiting its translation[4-6]. Currently, a large number of studies have shown that the expression levels of

Corresponding Author: Jiying Wang, MD; e-mail: jijuxiebaby@aliyun.com

miRNA family members are related to the metastasis and invasion of malignant tumors, such as lung cancer. The expression levels of some miRNAs are up-regulated in tumor development, thus playing a similar role as oncogenes. However, some miRNAs are down-regulated in tumor development, thus exerting the role of tumor suppressor genes. The abnormal expression of one miRNA molecule in the gene regulatory sequence can eventually affect the expression levels of hundreds of miRNAs. Therefore, miRNAs play a crucial role in the occurrence, development and progression of tumors[7-9].

As a member of the miRNA family, miR-520b has exhibited its unique advantages in the diagnosis and treatment of various malignant tumors, such as liver cancer[10], gastric cancer[11], ovarian cancer[12] and breast cancer[13]. However, few reports have explored the role of miR-520b in the occurrence and development of NSCLC. The aim of this study was to elucidate the role of miR-520b in the occurrence and development of NSCLC, and to explore the possible underlying mechanism.

Patients and Methods

NSCLC Patients and Cell Lines

A total of 50 NSCLC patients who received surgical procedure in Shanghai Pulmonary Hospital, Tongji University School of Medicine were enrolled in this study. All patients were confirmed NSCLC by pathological examinations. Preoperative chemotherapy or radiotherapy treatment was forbidden. Collected NSCLC tissues and corresponding adjacent normal tissues were kept in liquid nitrogen and a -70°C refrigerator, respectively. Adjacent normal tissues should be confirmed by biological biopsy to ensure that they did not include cancer cells. After all, Declaration of Helsinki should be mentioned and respected. This study was approved by the Ethics Committee of Shanghai Pulmonary Hospital, Tongji University School of Medicine. Signed written informed consents were obtained from all participants before the study. Human lung cancer cell line (A549), together with normal human lung epithelial cell LINE (BEAS-2B), were purchased from the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) complemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 IU/ml penicillin (Gibco, Rockville, MD, USA) and maintained in a 37°C, 5% CO_2 incubator.

Luciferase Reporter Gene Assay

Through searching the TargetScan, miRDB and microRNA websites, we found that chromatin assembly factor 1, subunit A (CHAF1A) was the target gene of miR-520b. The binding sequence of miR-520b at the 3'-end of CHAF1A was mutated by using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA). Subsequently, mutant CHAF1A (Mut-type) and non-mutant CHAF1A (WT-type) were connected with the pGL3-basic luciferase reporter vector (Promega, Madison, WI, USA). After lentivirus intervention on 24-well plates, PGL3-basic vector with mutant CHAF1A was transfected into A549 cells. The same treatment was performed on the pGL3-basic vector connected with non-mutant CHAF1A according to the instructions of the Luciferase Reporter Gene Assay Kit (Abnova, Taipei, Taiwan). Finally, luciferase activity was detected in a multi-function microplate reader.

Cell Transfection

MiR-520b mimics and si-CHAF1A were synthesized and transfected into NSCLC cells to analyze the biological function of miR-520b. Then, three groups were established to study the potential relevance between miR-520b and CHAF1A, including: the negative control group (NC), the miR-520b mimics group (A549 cells transfected with miR-520b mimics) and the mimics + CHAF1A group (A549 cells transfected with miR-520b mimics and si-CHAF1A). All the stuff was purchased form RiboBio (Guangzhou, China). Cell transfection was performed according to the instructions of Lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted under an RNase-free condition in strict accordance with the instructions of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). 2 μg total RNA were taken and diluted with 10.5 μL diethylpyrocarbonate (DEPC)-treated water (Beyotime, Shanghai, China). Next, 1 μL reverse transcription primer ligo-dT (500 μg/mL) was added, followed by water bath at 70°C for 5 min and at 4°C for 5 min. Subsequently, 4 μL 5 × Buffer, 2.5 μL deoxyribonucleoside tri-

phosphate (dNTP) (10 mmol/L), 0.5 μL RNase inhibitor and 0.5 μL Moloney murine leukemia virus (M-MLV) (200 U/μL) were added to make the final volume of 20 μL. After mixing evenly, the mixture was centrifuged, followed by reverse transcription reaction. The reverse transcription conditions were as follows: 42°C for 60 min, 70°C for 15 min, and storage at 4°C. QRT-PCR conditions were as follows: 95°C for 10 min, 95°C for 15 s, 60°C for 1 min, for a total of 40 cycles. Relative expression level of miR-520b was calculated by the $2^{-\Delta\Delta Ct}$ method, and U6 was used as an internal reference. Meanwhile, the expression level of CHAF1A was detected by qRT-PCR and endogenous controlled by GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

Western Blot Analysis

Radio-immunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China) was employed to extract total protein of A549 cells. The concentration of extracted protein was measured by the bicinchoninic acid (BCA) protein concentration kit (Pierce, Rockford, IL, USA). Equal amount of extracted proteins were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking with 5% skim milk, the membranes were incubated with rabbit anti-rat CHAF1A primary antibody (1:1000) at 4°C overnight. After fully washing with Tris-buffered saline with Tween-20 (TBST) (Beyotime, Shanghai, China), the membranes were incubated with anti-rabbit secondary antibody coupled by horseradish peroxidase (HRP) at room temperature for 2 h. Immuno-reactive bands were exposed by the enhanced chemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA). GAPDH was used as an internal reference, and relative changes of protein expression were calculated.

Cell Proliferation

When cells grew to the logarithmic growth phase, they were collected, diluted into 1×10^6 cell suspension and seeded into 96-well plates (5×10^3/100 μL per well). The wells only added with culture medium were used as blank controls. A total of 5 time points were set: including 0 (immediately after the cells were paved onto the plate and adhered to the wall), 24, 48, 72 and 96 h. Cell viability was determined *via* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 15 μL MTT reagent (500 μg/mL) were added into each well for culture for another 2 h. The absorbance (A) was then measured at 570 nm by an enzyme-labeled spectrophotometer, followed by zero setting using blank wells.

Cell Migration and Invasion

Migration assay: after the cells were counted, a total of 5×10^5 cells were added into the upper chamber. 500 μL serum-free medium were added into the upper chamber, whereas 800 μL Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS) were added into the lower chamber. After incubation for 12-18 h, the culture medium in both the upper and the lower chamber was discarded, and the filter membrane was washed with pre-heated phosphate-buffered saline (PBS). Meanwhile, PBS was gently blown and beaten to clean the lower surface of the filter membrane. Subsequently, 600 μL 4% paraformaldehyde were added into the lower chamber to fix the cells for 15 min. After removing the fixative, the transwell chamber was inverted to make the lower surface of filter membrane faced upwards. After drying naturally, the transwell chamber was stained with Giemsa at room temperature for 10 min. After washing with distilled water, cells not migrated on the surface were wiped off using a cotton ball, followed by observation under an inverted microscope. Finally, the number of migrated cells were counted and calculated. Invasion assay: matrigel was diluted to a final concentration of 1 mg/ml in 4°C pre-cooled serum-free medium. Then, 50 μL diluted Matrigel were added vertically to the center of the upper chamber, followed by incubation at 37°C for 0.5 h for gelatinization. The following steps were basically the same as migration assay.

Statistical Analysis

Prism 6.02 software (La Jolla, CA, USA) was used for all statistical analysis. Student's *t*-test or F-test was used to comparing the difference between different groups. All *p*-values were two-sided, and $p < 0.05$ were considered statistically significant.

Results

MiR-520b Expression was Decreased in Both NSCLC Tissues and Cell Lines

To explore the role of miR-520b in the development of NSCLC, we detected the expression

level of miR-520b in NSCLC tissues and adjacent normal tissues by qRT-PCR. Results showed that the expression of miR-520b in NSCLC tissues was significantly lower than that of adjacent normal tissues (Figure 1B). Liu et al[14] have found that CHAF1A is highly expressed in NSCLC. Therefore, we also detected the expression of CHAF1A in NSCLC tissues. As expected, the expression level of CHAF1A in NSCLC tissues was significantly up-regulated when compared with normal tissues (Figure 1A), which was consistent with the literature. Furthermore, same results were obtained in the cellular level (Figure 1C and 1D). Taken together, we thought that miR-520b correlate with CHAF1A during the progression of NSCLC.

CHAF1A was a Direct Target of miR-520b in NSCLC

To elucidate the putative and possible targets of miR-520b, we searched three publicly available algorithms, including TargetScan, miRDB and microRNA websites. Results showed that CHAF1A was predicted as a target gene of miR-520b (Figure 2A). Therefore, CHAF1A caught our attention and was implemented to our further studies. Firstly, we established luciferase reporter vectors containing wild- or mutant-type miR-520b seed sequences of the CHAF1A 3'-UTR. Results showed that transfection of miR-520b mimics significantly decreased the luciferase activity of wild-type CHAF1A 3'-UTR reporter gene. However, it

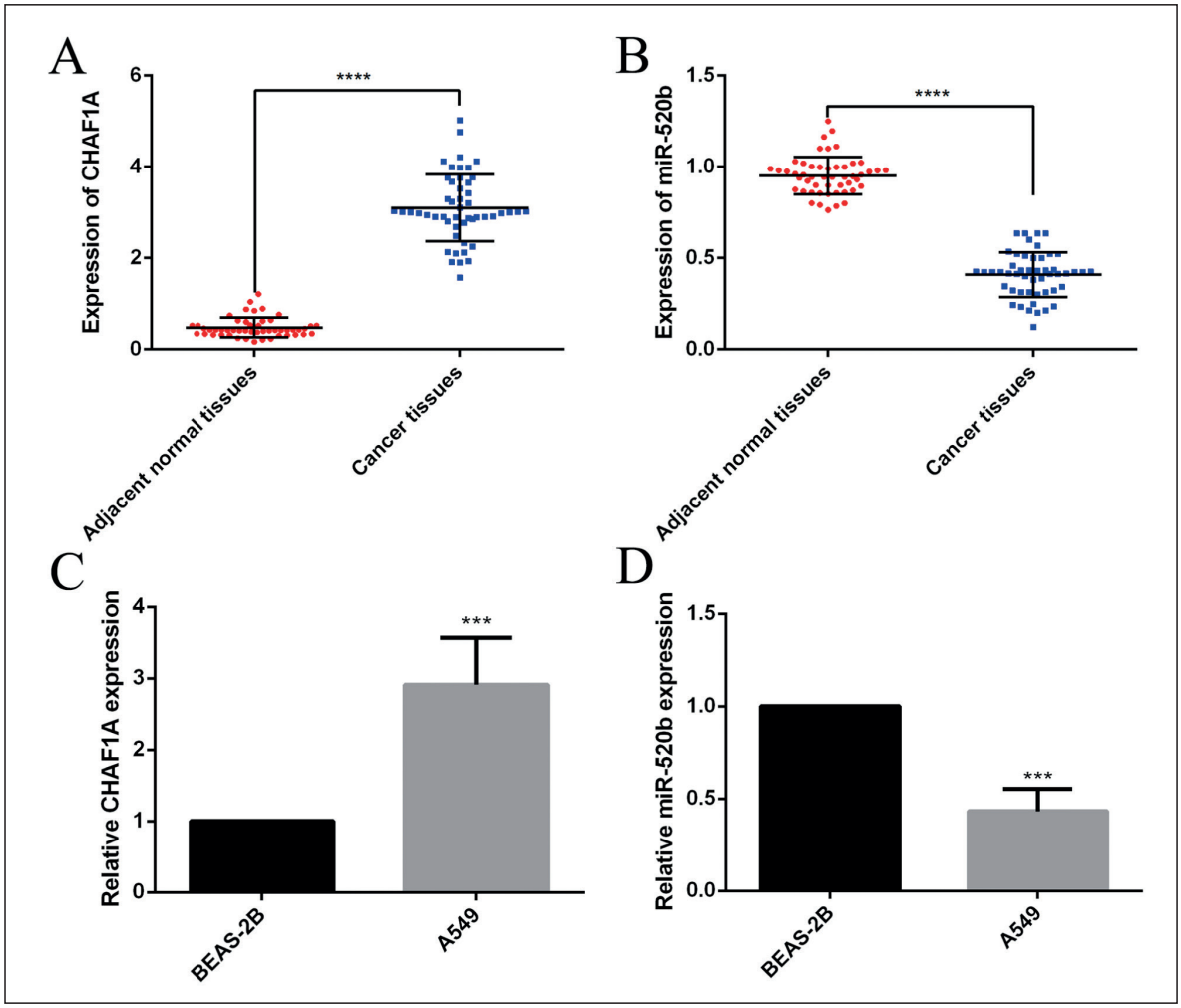

Figure 1. The expression levels of miR-520b and CHAF1A in NSCLC tissues and cells. ***A,*** and ***B,*** The expression of miR-520b and CHAF1A in NSCLC tissues and corresponding adjacent normal tissues. (****$p < 0.0001$ compared with adjacent normal tissues). ***C,*** and ***D,*** The expression of miR-520b and CHAF1A in NSCLC cells (A549) and normal human lung epithelial cells (BEAS-2B). (***$p < 0.001$ compared with BEAS-2B).

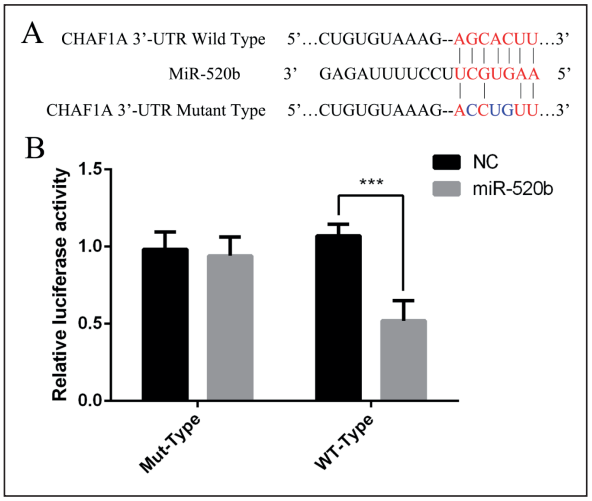

Figure 2. CHAF1A was a direct and functional target of miR-520b. A549 cells were transfected with miR-520b mimics and inhibitor. ***A,*** Diagram of putative miR-520b binding sites of CHAF1A. ***B,*** Relative activity of luciferase reporters (***$p < 0.01$).

had no effect on mutant-type (Figure 2B), suggesting that the expression of CHAF1A could be regulated by miR-520b.

MiR-520b Suppressed the Proliferation of NSCLC Cells

MTT assay was performed to examine the effect of miR-520b on the proliferation of NSCLC cells. Results showed that the proliferation rate of A549 cells was remarkably decreased by miR-520b mimics transfection. In contrast, down-regulated miR-520b significantly accelerated the growth of NSCLC cells (Figure 3).

MiR-520b Decreased the Expression Level of CHAF1A

Three groups were established in A549 cells for similar experiments, including the miR-NC group, the miR-520b mimics group and the mimics + CHAF1A group. QRT-PCR and Western blot experiment demonstrated that the expression level of CHAF1A was significantly decreased by miR-520b up-regulation in A549 cells (Figure 4A, 4B and 4C). These data further indicated that CHAF1A could be negatively regulated by miR-520b.

MiR-520b Inhibited Migration and Invasion of NSCLC Cells

Migration and invasion are two most important factors in the proliferation of cancer cells. Transwell assay indicated that the migration and invasion of A549 cells were significantly suppressed by the up-regulation of miR-520b. However, down-regulated miR-520b significantly enhanced the migration and invasion of NSCLC cells ($p < 0.05$) (Figure 4D, 4E and 4F).

Discussion

Lung cancer is one of the most common malignant tumors with highest morbidity and mortality in the world, seriously threatening human health. NSCLC accounts for 80-85% of all lung cancer patients[15]. With the rapid research of miRNAs in related fields, the treatment of NSCLC has entered the molecular era of multidisciplinary comprehensive treatment. Currently, multiple studies[16-18] have shown that miRNAs play a crucial role in the development and progression of malignant tumors, including lung cancer. As a gene expression regulator, miRNA is not only a noninvasive molecular marker for early diagnosis of various malignant tumors, but also helps clinicians to determine drug resistance and predict the prognosis of patients[19-22]. The role of miR-520b in malignant tumors has increasingly attracted more attention. The biological effect of miR-520b in different tumor

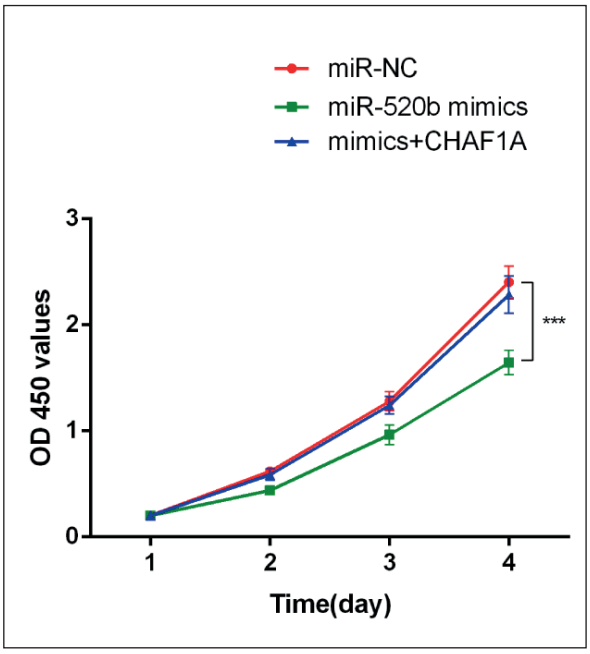

Figure 3. MiR-520b inhibited the proliferation of NSCLC cells. (***$p < 0.001$).

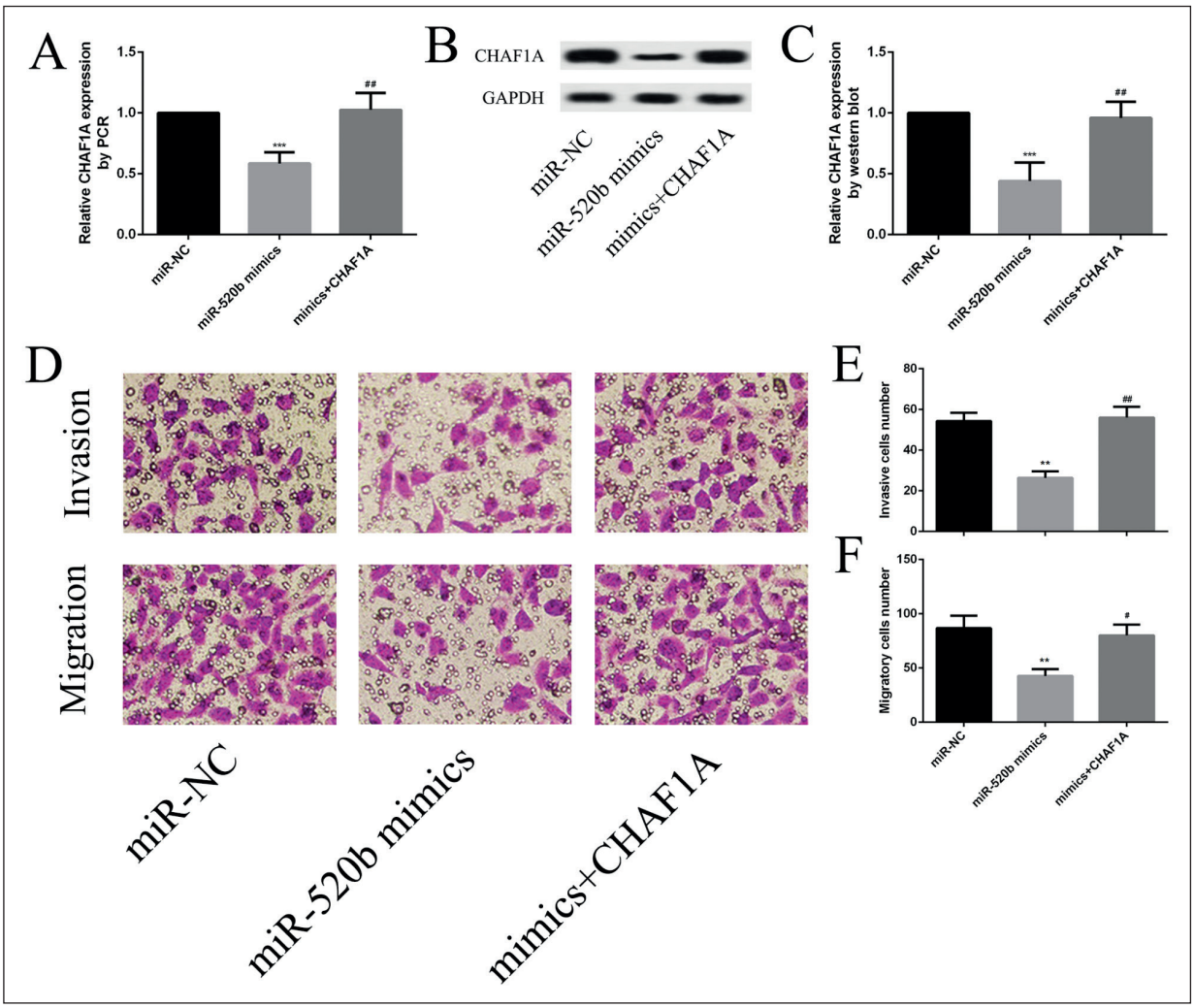

Figure 4. MiR-520b decreased the expression level of CHAF1A and inhibited the invasion and migration of NSCLC cells. ***A,*** Expression level of CHAF1A detected by qRT-PCR. ***B,*** Protein expression of CHAF1A detected by Western blot. ***C,*** Expression level of CHAF1A measured by Western blot. (**$p < 0.01$, ***$p < 0.001$ *vs.* the NC group; $^{\#\#}p < 0.01$ *vs.* the mimics group). ***D,*** The invasion and migration of NSCLC cells were analyzed by transwell assay. (200×). ***E,*** and ***F,*** statistical analysis of D. Data were presented as mean ± standard deviations. (**$p < 0.01$ *vs.* the NC group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* the mimics group).

cells differs. Researches have proved that the expression levels of miR-520b are significantly inhibited in gastric cancer and colon cancer. In the pathogenesis of the above cancers, miR-520b is considered as a tumor suppressor miRNA. Meanwhile, overexpression of miR-520b can reduce the proliferation, migration and invasion of tumor cells. The carcinogenic effect of miR-520b is exerted by regulating the expression of epidermal growth factor receptor (EGFR)[11] and DCUN1D1[23]. However, some other studies have indicated that the expression of miR-520b is significantly up-regulated in ovarian cancer. At the same time, miR-520b can promote the proliferation of tumor cells *via* mediating the expression of RING finger protein 216 (RNF216)[12]. However, the exact role of miR-520b in the development of NSCLC has not been fully elucidated. Recent investigations have shown that miRNA exerts its biological function *via* regulating target genes. Therefore, identifying target proteins is helpful to understand the molecular mechanism of miRNAs. MiR-520b has been confirmed to interact with multiple target genes. However, the mechanism of miR-520b in inhibiting lung cancer cells remains unclear. In this experiment, TargetScan, miRDB and microRNA websites were used to predict the possible

target genes of miR-520b. It was found that miR-520b could bind to CHAF1A. Luciferase reporter gene assay further confirmed that CHAF1A was a direct target gene of miR-520b. Besides, overexpression of miR-520b could significantly reduce the transcription and translation level of CHAF1A. As the main functional subunit of chromatin assembly factor-1 (CAF-1) protein, CHAF1A is involved in deoxyribonucleic acid (DNA) replication, gene expression regulation and DNA mismatch repair[24-28]. With the deepening research of CHAF1A, it has been found that CHAF1A plays an important role in promoting the occurrence and development of malignant tumors. More and more studies have shown that CHAF1A exhibits an important relationship with the formation and prognosis of various tumors. Meanwhile, overexpression of CHAF1A gene can significantly increase the proliferation, migration and invasion of tumor cells[29-31]. Therefore, deeply studying the role of CHAF1A in the development of tumor is of great significance for the diagnosis and treatment of tumors. In NSCLC, the expression of CHAF1A has been found to be significantly up-regulated[14]. Moreover, inhibiting CHAF1A has also been proved to suppress the proliferation of lung cancer cells. These studies indicate that CHAF1A acts as an oncogene in NSCLC. Our study demonstrated that CHAF1A was a direct target of miR-520b in NSCLC. At the same time, it was also found that the expression of CHAF1A in lung cancer tissues and cells was significantly up-regulated. CHAF1A expression was negatively correlated with the expression of miR-520b, which was consistent with previous studies. It was noteworthy that overexpression of CHAF1A could effectively reverse the inhibitory effect of miR-520b on the proliferation, migration and invasion of NSCLC cells. These results all indicated that the inhibitory effect of miR-520b on NSCLC was achieved by inhibiting CHAF1A.

Conclusions

We found that the expression of miR-520b was significantly decreased in NSCLC. Moreover, overexpression of miR-520b could significantly inhibit the proliferation, invasion and metastasis of tumor cells, which was achieved by inhibiting CHAF1A expression. This study might provide a potential therapeutic strategy for the treatment of NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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