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MicroRNA-495 inhibits the progression of non-small-cell lung cancer by targeting TCF4 and inactivating Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: Different effects of microRNA-495 (miR-495) on human cancers have been exhibited in recent years. However, the specific function of miR-495 remains uncertain in non-small-cell lung cancer (NSCLC). Thus, we aim to explore the role of miR-495 in NSCLC.

PATIENTS AND METHODS: The expressions of miR-495 and transcription factor 4 (TCF4) were detected through quantitative Real-time polymerase chain reaction (qRT-PCR) assay. Western blot was used to measure the protein expression of relative genes. The relationship between miR-495 and TCF4 was testified by the dual-luciferase reporter gene assay. The function of miR-495 was investigated through cell counting kit-8 (CCK-8) assay and transwell assay.

RESULTS: MiR-495 was downregulated in NSCLC tissues. Overexpression of miR-495 inhibited the migration, invasion and proliferation of NSCLC cells. Further, TCF4 was a direct target gene of miR-495. TCF4 was highly expressed in NSCLC tissues. In addition, miR-495 inhibited the progression of NSCLC through targeting TCF4. Furthermore, miR-495 inhibited EMT and Wnt/β-catenin pathway in NSCLC.

CONCLUSIONS: MiR-495 inhibited the progression of NSCLC by targeting TCF4 and inactivating Wnt/β-catenin pathway.

Key Words:
miR-495, Non-small-cell lung cancer, TCF4, Wnt/β-catenin pathway.

Introduction

Lung cancer has become the leading death of malignant tumor in Chinese urban population[1]. Moreover, about 80% of lung cancer cases belong to non-small cell lung cancer (NSCLC). About 75% of NSCLC patients are in the middle and late stage at the first time of diagnosis, with a very low 5-year survival rate[2]. Furthermore, postoperative recurrence is most prominent in the treatment of NSCLC. NSCLC patients have spread metastasis when diagnosing[3]. Metastasis is an important and complicated multistep in human cancers, including cell migration, invasion and epithelial mesenchymal transition (EMT)[4]. EMT has been paid more attention because of its close correlation with distal metastasis and invasion[5]. The transform from epithelial cells to mesenchymal-like cells is the main characteristic for EMT. During EMT, the cancer cells acquire the properties of migration and invasion[6]. Previous studies[7] reported that EMT can be regulated by the several signal pathways, such as Wnt/β-catenin signaling. Wnt/β-catenin signaling pathway could promote EMT in gastric cancer by epigenetic regulation[8]. Therefore, the regulatory mechanism underlying EMT and Wnt/β-catenin pathway in NSCLC is investigated here. MicroRNAs (miRNAs) have become the focus of attention due to their crucial effect in cancers[9]. Previous studies also indicated that miRNAs regulate many biological activities of NSCLC, such as growth, proliferation, metastasis, and apoptosis. For example, miR-550a-3p promotes proliferation and metastasis of NSCLC cells through down-regulating TIMP2[10]. Yang et al[11] demonstrated that miR-598 suppressed invasion and migration by negative regulation of Derlin-1 and EMT in NSCLC. In all of these miRNAs, the function of miR-495 associated with tumorigenesis and development of human cancers attracts our attention. Moreover, the contradictory effect of miR-495 has been reported in several human cancers. Tan et al[12] proposed that miR-495 promoted growth and invasion of bladder cancer cells by targeting phos-

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phatase and tensin homolog. Oppositely, Yan et al[13] suggested that miR-495 suppressed proliferation and migration of colorectal cancer cells by targeting FAM83D. Moreover, miR-495 has been reported to enhance the sensitivity of NSCLC cells to platinum by regulating ATP7A[14]. To date, the function of miR-495 associated with the development of NSCLC is still unclear. In addition, transcription factor 4 (TCF4) has been found to promote occurrence and development of several cancers as an oncogene. Moreover, TCF4 can specifically recognize β-catenin using alternative conformations to be involved in Wnt/β-catenin signaling pathway[15]. Regulatory region of metastasis-inducing DNA is the binding site for TCF4[16]. Moreover, TCF4 can inhibit the metastatic development through regulating the expressions of endogenous genes[16]. In this study, we investigated the relationship between miR-495 and TCF4 in NSCLC. The potential biological function of miR-495 in regulating the development of NSCLC was confirmed as well. Meantime, the effect of miR-495 on EMT and Wnt/β-catenin signaling pathway in NSCLC was identified. However, further evaluation is still needed to explore whether miR-495 can act as a biomarker for the diagnosis and therapy of NSCLC.

Patients and Methods

Clinical Tissues

Sixty-four paired human NSCLC tissues and adjacent normal lung tissues were acquired from the People's Hospital of Dezhou. All enrolled NSCLC patients did not receive any treatment before the operation and they signed the informed consent. These tissues were frozen in liquid nitrogen and then stored in the -80°C refrigerator. This experiment was approved by the Institutional Ethics Committee of People's Hospital of Dezhou.

Cell Lines Culture

H1299, A549 and H1650 cell lines and a normal human bronchial epithelial cell line (BEAS-2B) were used for this experiment. These cell lines were acquired from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and were maintained at 37°C with 5% CO_2.

Cell Transfection

The miR-495 mimics, inhibitor and negative control (NC) were obtained from GenePharma (Shanghai, China). Plasmids were transfected in A549 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufactures' protocols.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied to extract total RNA in NSCLC. Synthesis of complementary deoxyribose nucleic acid (cDNA) was performed using a RevertAid RT Transcription Kit (Thermo Fisher, Waltham, MA, USA). We conducted qRT-PCR through using SYBR Green Reagent (Thermo Fisher, Waltham, MA, USA) on ABI 7500 Fast system (Applied Biosystems, Foster City, CA, USA). U6 or GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as control for miR-495 or TCF4, respectively, and their expressions were calculated using the $2^{-\Delta\Delta ct}$ method.

Western Blot Analysis

The protein samples were obtained using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Proteins were separated through a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were incubated with 5% skim milk at room temperature. After that, we incubated the membranes overnight at 4°C with EMT markers (E-cadherin, N-cadherin, vimentin), Wnt/β-catenin pathway markers (β-catenin), TCF4 and GAPDH antibodies. After washing, they were incubated with corresponding secondary antibodies for 2 h at room temperature. Then, protein expression levels were measured by electrochemiluminescence (ECL, Pierce Biotechnology, Rockford, IL, USA).

Cell Counting Kit-8 (CCK-8) Assay

CCK-8 assay was performed to measure cell proliferation based on the manufacturer's instructions. A549 cells were seeded in 96-well plates with 5×10^4 cells per well and incubated for 0, 24, 48 and 72 h. Cells were maintained in an incubator with 5% CO_2 at 37°C. Next, 10 μL CCK-8 reagents were added in each well for 2-h-incubation (Dojindo, Tokyo, Japan). Finally,

optical density was detected using a microplate reader (Molecular Devices) at the absorbance of 450 nm.

Transwell Assay

Transwell chambers (8-μm pore size membranes) were employed to perform cell migration and invasion assays. The lower chamber was added with 10% FBS and incubated at 37°C with 5% CO_2. The upper surface coated with matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was used for cell invasion. Cell migration assay was conducted without coating matrigel in the chamber. A549 cells transfected with miR-495 mimic or inhibitor were cultured in the upper chamber with serum-free medium. 48 h later, the migrated or invasive cells were fixed with methanol and stained with crystal violet. Finally, we counted the number of penetrating cells using a microscope.

Dual-Luciferase Reporter Gene Assay

The wild or mutant type of 3'-UTR of TCF4 was inserted into pcDNA3.1 plasmid vector (Promega, Madison, WI, USA) to perform luciferase reporter experiments. Then, wild or mutant type of 3'-UTR of TCF4 and miR-495 mimic were co-transfected into A549 cells. Subsequently, the luciferase activity was measured through dual-luciferase assay system (Promega, Madison, WI, USA).

Statistical Analysis

The data were shown as mean ± SD. Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) or Graphpad Prism 6 (La Jolla, CA, USA) was employed to analyze these data. The correlation of miR-495 expression with clinic pathological characteristics of NSCLC patients was evaluated through the x^2-test. The difference between groups was calculated through one-way ANOVA, followed by Post-Hoc Test (Least Significant Difference). The survival curves were draw by Kaplan-Meier analysis, and log-rank test was used to compare the survival differences. Significant difference was defined at $p < 0.05$.

Results

MiR-495 Was Downregulated in NSCLC Tissues

The expression levels of miR-495 were firstly detected in NSCLC tissues by qRT-PCR assay. MiR-495 expression significantly decreased in NSCLC tissues in contrast to the normal tissues (Figure 1A). x^2 analysis showed the abnormal expression of miR-495 was correlated with lymph nodes metastasis ($p = 0.021$) and tumor stage of NSCLC patients ($p = 0.005$, Table I). In addition, lower expression of miR-495 was associated with shorter overall survival of NSCLC patients ($p = 0.0243$, Figure 1B). Based on these results, we considered that miR-495 might play an important role in the pathogenesis of NSCLC.

MiR-495 Inhibited the Migration, Invasion and Proliferation of NSCLC Cells

We observed the expression of miR-495 in H1299, A549, H1650 and BEAS-2B cell lines.

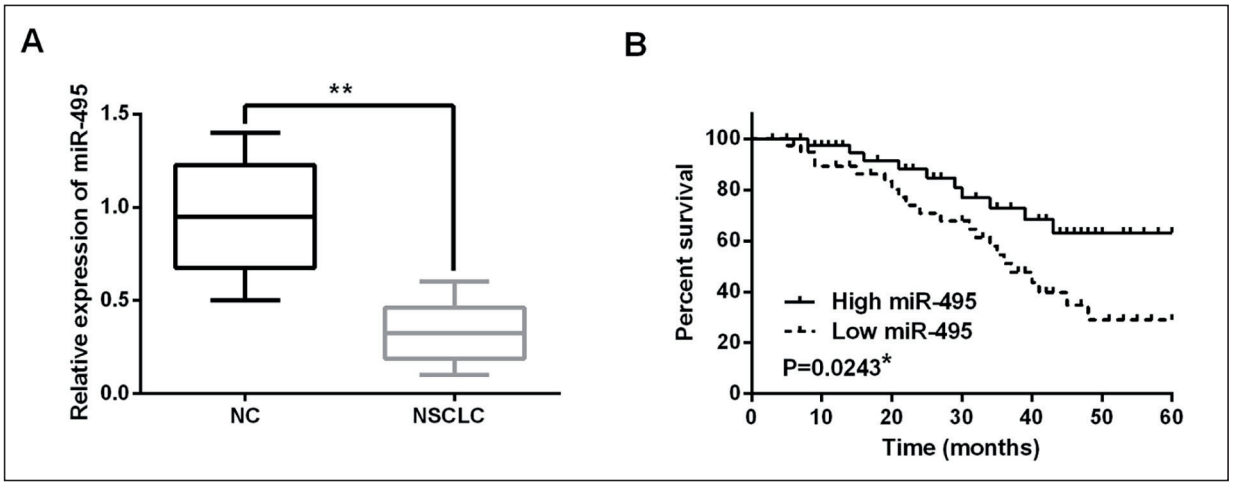

Figure 1. MiR-495 was downregulated in NSCLC tissues. ***A,*** The expressions of miR-495 in NSCLC tissues. ***B,*** Lower miR-495 expression was correlated with shorter overall survival of NSCLC patients. *$p < 0.05$, **$p < 0.01$.

Table I. Relationship between miR-495 expression and their clinic-pathological characteristics of NSCLC patients.

Characteristics	Cases	miR-495		*p*-value
		Low	High	
Age (years)				0.094
≥ 60	44	18	26	
< 60	20	9	11	
Gender				0.166
Male	40	13	27	
Female	24	9	15	
Tumor size (mm)				0.792
≤ 3	34	10	24	
> 3	30	12	18	
Lymph nodes metastasis				0.021*
Yes	10	3	7	
No	54	15	39	
Tumor stage				0.005*
I-II	46	20	26	
III-IV	18	8	10	

Statistical analyses were performed by the χ^2 test. *$p < 0.05$ was considered significant.

Similarly, downregulation of miR-495 was identified in H1299, A549 and H1650 cell lines in comparison to BEAS-2B cells (Figure 2A). Then, miR-495 mimics or inhibitor was transfected into A549 cells to investigate its function in NSCLC. MiR-495 mimics transfection apparently increased the expression of miR-495 in NSCLC cells. Oppositely, miR-495 inhibitor transfection reduced its expression in A549 cells (Figure 2B). Next, overexpression of miR-495 was found to inhibit the proliferation of A549 cells (Figure 2C). Knockout of miR-495 promoted cell proliferation in NSCLC (Figure 2D). Similarly, the migration of A549 cells was suppressed by upregulation of miR-495 and promoted by miR-495 knockdown (Figure 2E). In the meantime, the same results were also identified for cell invasion in A549 cells (Figure 2F). Taken together, we considered that miR-495 is a tumor suppressor in the development of NSCLC.

TCF4 Was a Direct Target Gene of miR-495

Further, TCF4 was selected as a target gene of miR-495. MiR-495 can bind with the 3'-UTR of TCF4 predicted by TargetScan (http://www.targetscan.org/) (Figure 3A). Then luciferase reporter was performed to testify that prediction. We found that the luciferase activity of Wt-TCF4 was significantly reduced by transfection of miR-495 mimics. However, the luciferase activity of Mut-TCF4 was not affected by miR-495 mimics (Figure 3B). Moreover, a negative correlation was found between expressions of TCF4 and miR-495 in NSCLC tissues ($p < 0.001$, $R^2 = 0.4507$; Figure 3C). To further confirm that correlation, TCF4 expression was measured in A549 cells after transfection of miR-495 mimics or inhibitor. The expression of TCF4 was declined by transfection of miR-495 mimics (Figure 3D) and promoted by transfection of miR-495 inhibitor (Figure 3E). These results confirmed that TCF4 is a direct target gene of miR-495. A negative association was existed between TCF4 and miR-495 in NSCLC tissues.

TCF4 Was Upregulated in NSCLC Tissues

Subsequently, the alternation of TCF4 expression was identified in NSCLC tissues and cell lines. Increased expression of TCF4 was identified in NSCLC tissues compared with the adjacent normal tissues (Figure 4A). Upregulated TCF4 was also identified in H1299, A549 and H1650 cell lines in contrast to the BEAS-2B cells (Figure 4B). Moreover, we found that higher expression of TCF4 was associated with shorter overall survival of NSCLC patients (p=0.0118, Figure 4C). Hence, TCF4 was inferred to participate in the development of NSCLC.

MiR-495 Inhibited the Progression of NSCLC Through Targeting TCF4

Next, miR-495 mimics and TCF4 vector were co-transfected into A549 cells to confirm their interaction. QRT-PCR assay showed that the reduction of TCF4 expression regulated by miR-

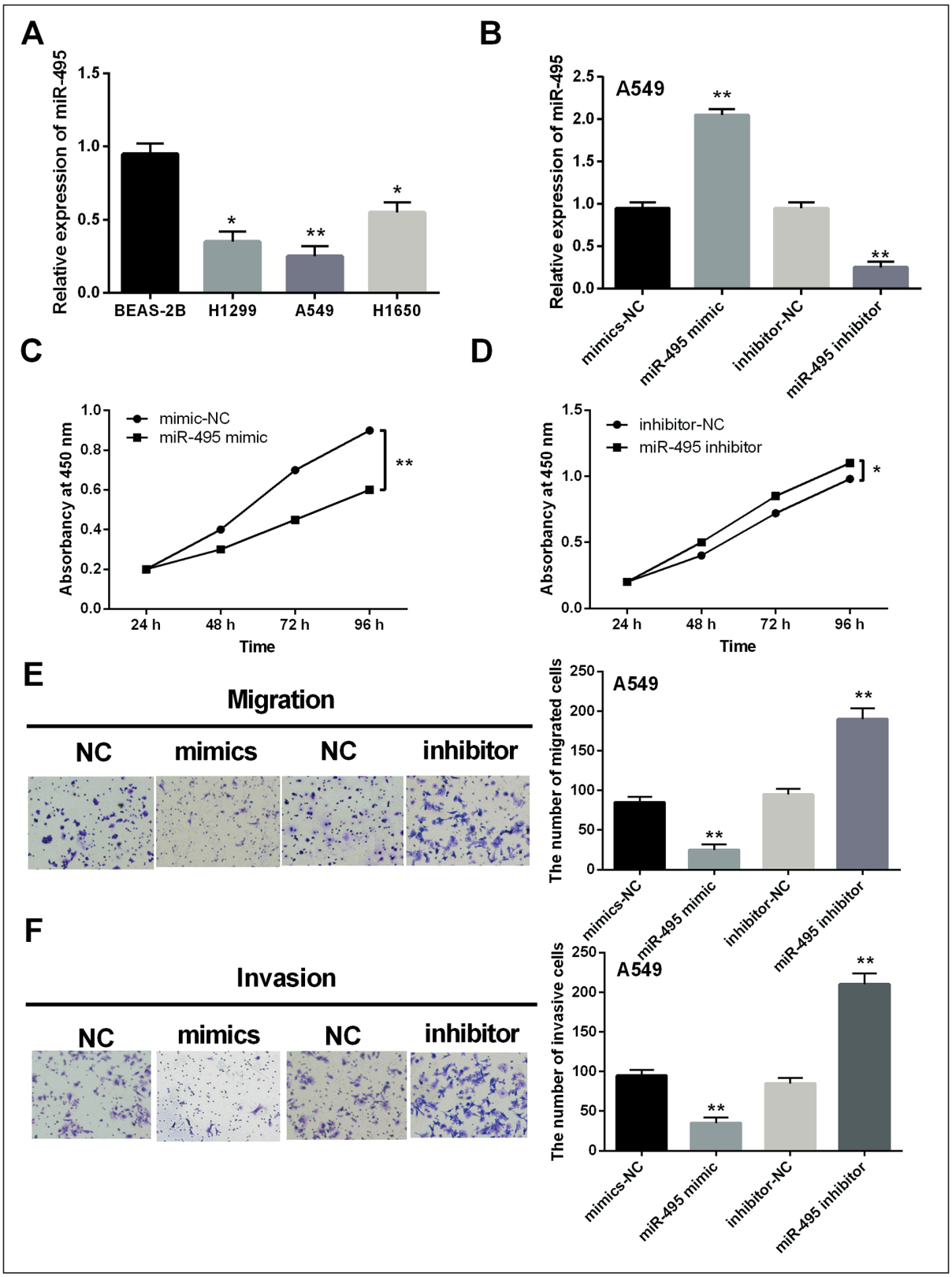

Figure 2. MiR-495 inhibited the migration, invasion and proliferation of NSCLC cells. ***A,*** MiR-495 expression in H1299, A549, H1650 and BEAS-2B cell lines. ***B,*** The expression of miR-495 was examined in A549 cells with transfection of miR-495 mimics or inhibitor. ***C, D,*** The cell proliferation was measured in cells transfected with miR-495 mimics or inhibitor. ***E, F,*** Cell migration and invasion analysis in cells transfected with miR-495 mimics or inhibitor *$p < 0.05$, **$p < 0.01$.

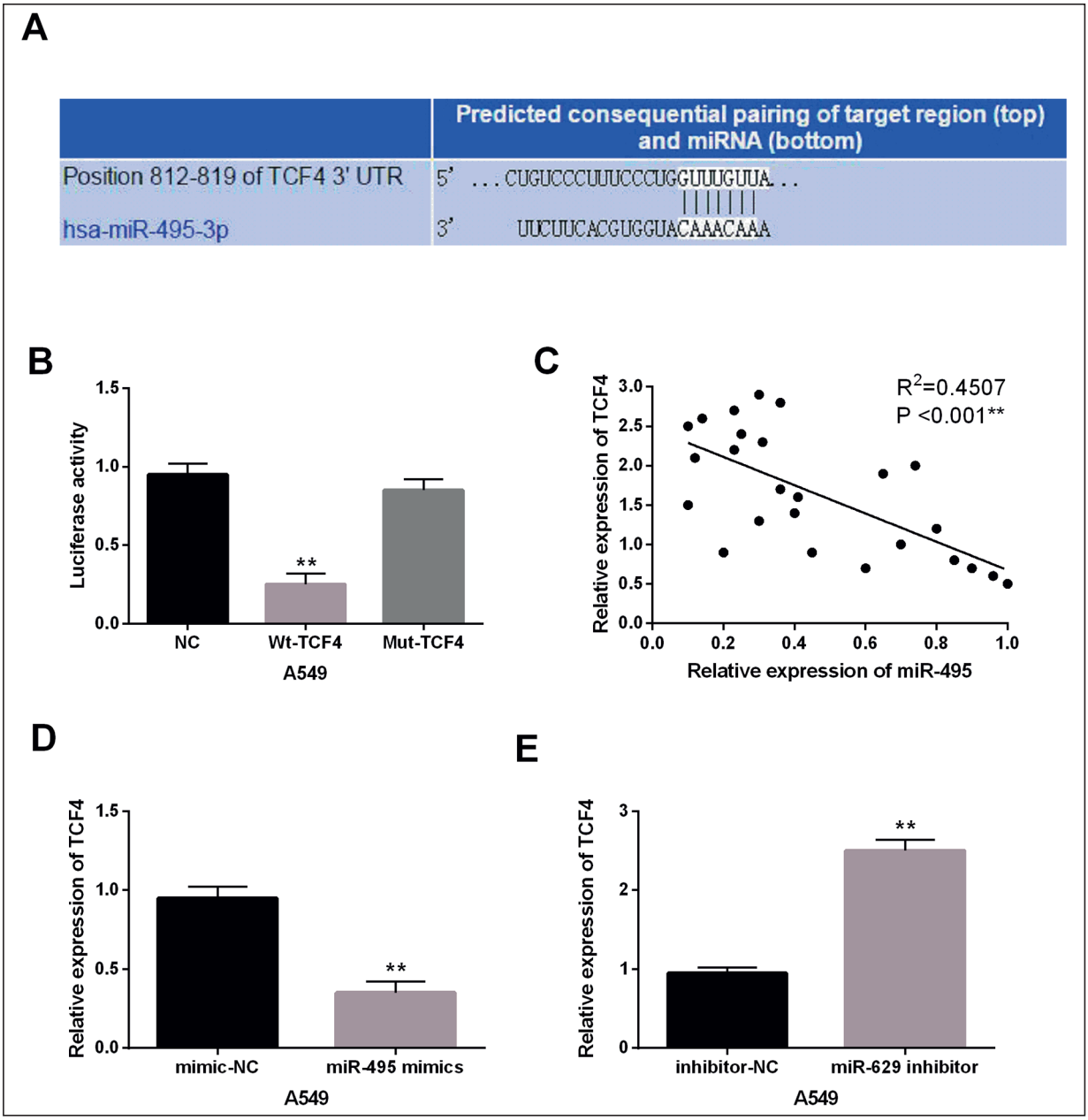

Figure 3. TCF4 was a direct target gene of miR-495. ***A,*** TCF4 had binding sites with miR-495. ***B,*** Dual-luciferase reporter gene assay. ***C,*** Negative correlation between miR-486 and TCF4. ***D, E,*** The expression of TCF4 in A549 cells transfected with miR-495 mimics or inhibitor $^{**}p < 0.01$.

495 mimics was restored by transfection of TCF4 vector in A549 cells (Figure 5A). Similarly, the inhibitory effect of miR-495 on cell proliferation in NSCLC was hindered by transfection of TCF4 vector as well (Figure 5B). Moreover, the same results were identified for cell migration and invasion in NSCLC (Figure 5C, 5D). To sum up, miR-495 inhibited the progression of NSCLC through targeting TCF4.

MiR-495 Repressed EMT and Wnt/β-Catenin Pathway in NSCLC

Finally, the effect of miR-495 on EMT and Wnt/β-catenin pathway was investigated in NSCLC. Firstly, overexpression of miR-495 inhibited the expressions of N-cadherin and Vimentin and promoted E-cadherin expression in A549 cells (Figure 6A). Inversely, the opposite effect of miR-495 silence was observed in the altered expressions

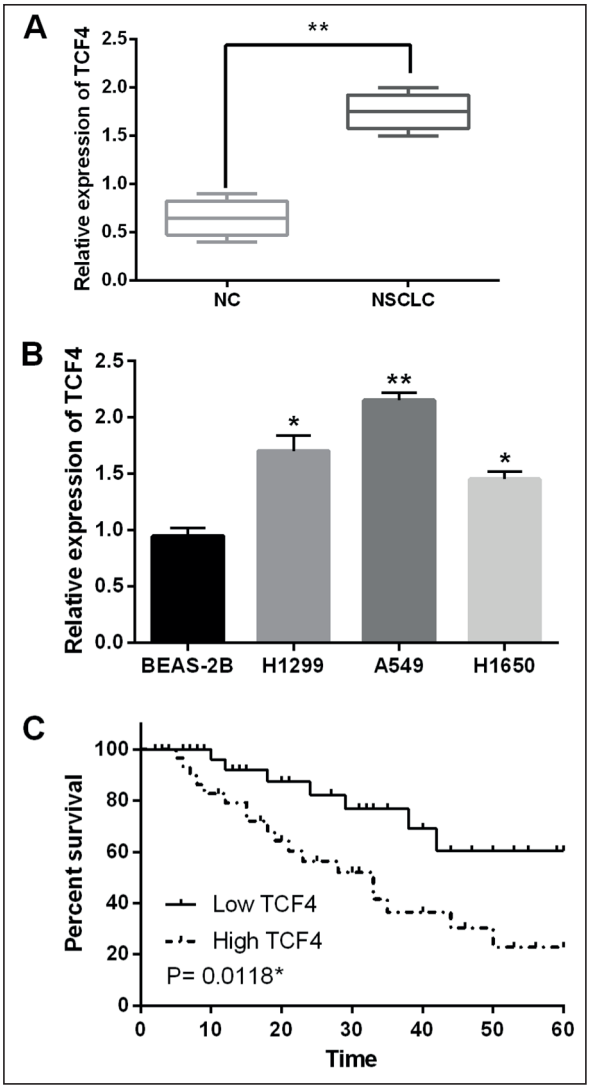

Figure 4. TCF4 was upregulated in NSCLC tissues. ***A,*** The expressions of TCF4 in NSCLC tissues. ***B,*** The TCF4 expression in H1299, A549, H1650 and BEAS-2B cell lines. ***C,*** Higher TCF4 expression was related to shorter overall survival of NSCLC patients. *$p < 0.05$, **$p < 0.01$.

of three markers (Figure 6B). As for Wnt/β-catenin pathway, overexpression of miR-495 was identified to block the expression of β-catenin (Figure 6A) while miR-495 knockdown promoted β-catenin expression (Figure 6B). Therefore, miR-495 repressed EMT and Wnt/β-catenin pathway to impede the progression of NSCLC.

Discussion

Researches confirmed that the progression of malignant tumors was closely associated with miRNAs. There are many miRNAs have been reported to participate in the pathogenesis of NSCLC. Qin et al[17] proposed that miR-340 was downregulated in NSCLC and lower miR-340 expression predicted worse prognosis of NSCLC. Here, we found that miR-495 was downregulated in NSCLC. Lower expression of miR-495 indicated a worse prognosis of NSCLC. Functionally, Yang et al[18] reported that upregulation of miR-4299 suppressed proliferation, migration and invasion of NSCLC cells. In this study, overexpression of miR-495 inhibited the migration, invasion and proliferation of NSCLC cells. In addition, miR-495 was found to repress EMT and Wnt/β-catenin pathway in NSCLC. These findings indicated that miR-495 was a suppressive miRNA in NSCLC development. The abnormal expression and function of miR-495 have been identified in some human cancers. Same as our results, downregulated miR-495 had been detected in hepatocellular carcinoma[19], esophageal squamous cell carcinoma[20] and gastric cancer[21]. Besides, we performed *in vitro* experiments to investigate the biological effect of miR-495 on NSCLC cells. The proliferation, migration, invasion of NSCLC cells were inhibited by upregulation of miR-495. Consistent with our findings, Chu et al[22] also demonstrated that miR-495 repressed proliferation and migration in NSCLC by targeting MTA3. In addition, miR-495 had been found to promote the chemoresistance of small cell lung cancer (SCLC) through the EMT *via* ETK/BMX[23]. All these results suggested that miR-495 was an important tumor-suppressor gene in NSCLC. Further, the relationship between miR-495 and TCF4 was testified in present study to explore the regulatory mechanism of miR-495 in NSCLC. In different cancers, previous studies have proposed several direct genes of miR-495, such as Bmi-1[24], CDK6[25] and HMGA2[26]. It was firstly reported that miR-495 directly targeted TCF4. Through our experiments, miR-495 inhibited the progression of NSCLC by targeting TCF4. Gu et al[27] also demonstrated that miR-139 suppressed the proliferation and invasion of hepatocellular carcinoma through the Wnt/TCF4 pathway. The Wnt/β-catenin pathway regulated by miR-495 was also explored in NSCLC. Moreover, the Wnt/β-catenin pathway has been reported to be involved in the tumorigenesis of human cancers[28, 29]. Chen et al[30] revealed that miR-24 inhibited the proliferation and invasion of glioma *via* β-catenin/TCF4 signaling pathway. Here, miR-495 was also found to inhibit the progression of NSCLC by impeding TCF4/β-catenin pathway.

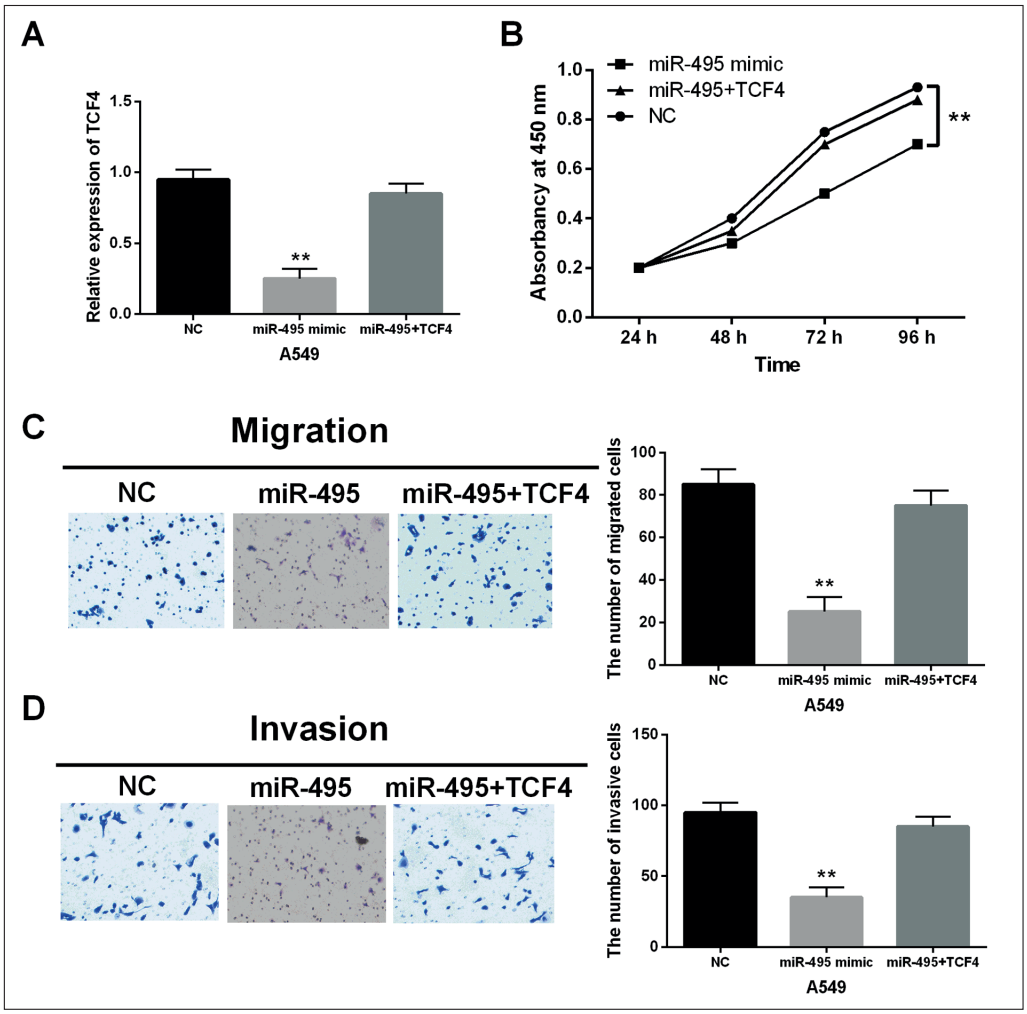

Figure 5. MiR-495 inhibited the progression of NSCLC through targeting TCF4. ***A,*** The expression of TCF4 was measured in A549 cells transfected with TCF4 vector and miR-495 mimics. ***B,*** The cell proliferation was measured in A549 cells transfected with TCF4 vector and miR-495 mimics. ***C, D,*** The cell migration and invasion in A549 cells transfected with TCF4 vector and miR-495 mimics $^{**}p < 0.01$.

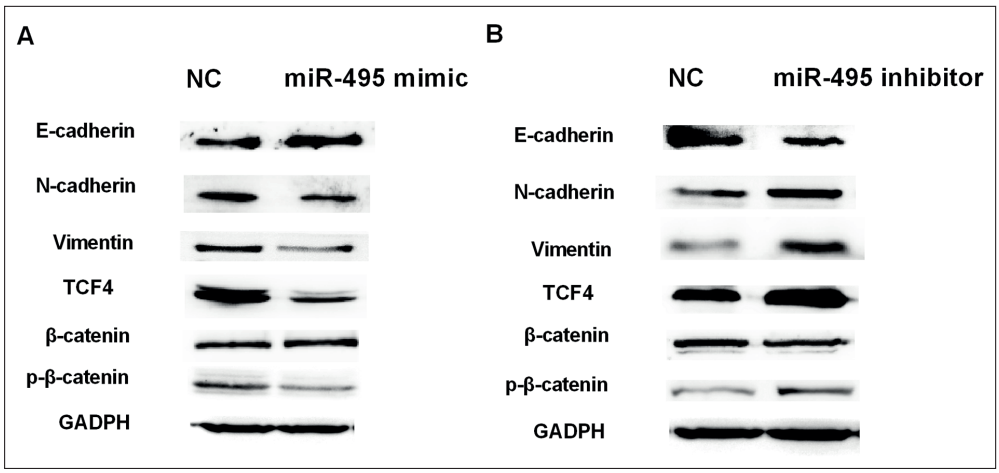

Figure 6. MiR-495 repressed EMT and Wnt/β-catenin pathway in NSCLC. ***A, B,*** Western blot analysis of protein expressions of E-cadherin, N-cadherin, Vimentin and β-catenin in A549 cells transfected with miR-495 mimics or inhibitor.

Conclusions

miR-495 was downregulated in NSCLC and its lower expression was related to shorter overall survival of NSCLC patients. More importantly, miR-495 inhibited the migration, invasion and proliferation of NSCLC cells through targeting TCF4 and inactivating Wnt/β-catenin pathway. These findings implied that miR-495 might be a valuable biomarker for the diagnosis and therapies of NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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