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Lncrna TUBA4B Inhibits Cell Migration and Invasion via Mir-127 in Non-Small Cell Lung Cancer

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Research Article

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ABSTRACT

Long non-coding RNA (lncRNA) is an important mediator involved in cancer progression. Previous studies have confirmed that TUBA4B is low expressed in tumor diseases. The potential impact of TUBA4B on the progression of non-small cell lung cancer remains unknown. In this article, TUBA4B was found to be down-regulated in non-small cell lung cancer tissues, and its levels are related to the stage of non-small cell lung cancer, tumor size, and lymph node metastasis. Further experiments showed that overexpression of TUBA4B in A549 cells inhibited the invasion and migration of non-small cell lung cancer cells. TUBA4B regulates the expression of miR-127, and the expression of TUBA4B is negatively correlated with miR-127 in non-small cell lung cancer tissues. Rescue tests confirmed that TUBA4B regulates cell invasion and migration through miR-127 in non-small cell lung cancer. Overall, our results suggest that the TUBA4B/miR-127 axis affects the progression of non-small cell lung cancer and provides a potential new target for patients with non-small cell lung cancer.

INTRODUCTION

Non-small cell lung cancer (NSCLN), accounts for approximately 85% ~ 90% of primary lung cancer, the leading cause of cancer-related mortality worldwide [1,2]. Despite great improvements in research and optimization of treatment strategies, two-thirds of non-small cell lung cancer are diagnosed in advanced stage, resulting in a poor 5-year survival [3]. The prognosis would be greatly improved if lung cancer is diagnosed at early stage. Thus, it is urgent to investigate the biomarkers for early diagnosis of lung cancer.

Long non-coding RNAs (lncRNAs) represent a category of non-coding RNAs with length over 200 nucleotides, play significant roles in multiple fundamental biological processes [4,5]. Recently, lncRNAs have emerged as new hotspots for the treatment and diagnosis of multiple diseases, especially in malignant tumors [6]. Deregulated lncRNAs were reported in a variety of malignant tumors including hepatocellular carcinoma, bladder cancer, breast cancer, osteosarcoma and colorectal cancer [7-11]. For example, LncRNA TUBA4B acted as a competitive endogenous RNA (ceRNA) via sponging miR-214 and miR-216a/b to inhibit gastric cancer progression [12]. LncRNA GAS5 via sponging miR-222 to inhibit colorectal cancer cell migration and invasion, and promote autophagy [13].

Particularly, lncRNA was involved in biological processes by competitive binding microRNA (miRNAs). miRNAs are another kinds of non-coding RNAs that has 18-25 nucleotides in length [14]. Increasing evidences demonstrated that miRNAs could inhibit gene expression via directly targeting the 3'-untranslated region (3'-UTR) of target mRNAs [15]. miR-127 promoted chondrogenic differentiation in rat bone marrow mesenchymal stem cells [16]. miR-127 was upregulated in osteosarcoma cells, and enhanced cell viability, migration, and invasion and inhibited cell apoptosis [17]. However, miR-127 acted as a tumor suppressor and down regulated in epithelial ovarian cancer, esophageal squamous cell carcinoma and also in ovarian cancer [18-20]. However, the

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functions of miR-127 in non-small cell lung cancer are still unknown.

In this study, we mainly explore the expression characteristics and biological functions of TUBA4B in the progression of non-small cell lung cancer. As a miR-127 sponge, TUBA4B regulates the cell invasion and migration ability of non-small cell lung cancer. Our research provides a potential new direction for the future development of targeted therapy for non-small cell lung cancer.

MATERIALS AND METHODS

Sample collection

During January 2015 to December 2018, 49 non-small cell lung cancer patients were collected from the first affiliated hospital of Shandong first medical university, and we obtained 49 pairs of NSCLN tissue samples and corresponding precancerous tissue samples through surgical operation. NSCLN tissues were pathologically confirmed by at least two experienced pathologists based on the WHO classification criteria. After surgical resection, all the tissues were rapidly frozen in liquid nitrogen and stored at -80°C. All the informed consents were obtained before this study, and the scheme was approved by the Ethics Committee of the first affiliated hospital of Shandong first medical university.

Cell culture

Human NSCLC cell lines (A549, PC9 and HCC827) and a normal human bronchial epithelial cell line HBE were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All the cells were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) at 37°C in a humidified incubator with 5% CO2.

Cell transfection

pGL4-TUBA4B, miR-127 mimic, miR-127 inhibitor and control were synthesized and purchased from GenePharma (Shanghai, China). A549 cells were seeded in 6-well plat, and cultured to the confluence of 70%. Cells were transfected using the Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) according to the instructions. Lipofectamine 2000 and serum-free medium were mixed and placed in a sterile Eppendorf (EP) tube for 5 minutes. Meanwhile, mix the vector and serum-free medium into another sterile EP tube. The solution in the above two test tubes was mixed and allowed to stand at room temperature for 20 minutes to obtain a complex of RNA and liposomes. This mixture was added to a petri dish containing cells to be transfected, and cultured at 37°C with 5% CO2.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were isolated and lysed from cells or tissues using the TRIzol reagent (Thermo Fisher Scientific). The reverse transcription was performed to synthetize the first cDNA chain by using the PrimeScript RT Reagent Kit (Takara, Otsu, Japan). Subsequently, real-time quantitative polymerase chain reaction (RT-qPCR) with SYBR Green qPCR mix kit (Takara, Kyoto, Japan) to quantify relative levels of mRNAs and miRNAs. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 are used as internal references for TUBA4B and miR-127, respectively.

Dual-luciferase reporter assay

Based on the binding site predicted by Starbase, wild-type and mutant sequences of TUBA4B were inserted into the pGL4 vector to construct TUBA4B wild-type (TUBA4B-WT) and TUBA4B mutant (TUBA4B-MT). Cells were co-transfected with wild type / mutant vector and miR-127 mimic / NC using Lipofectamine 2000. Promega (Madison) dual luciferase reporter detection system was used to determine luciferase activity.

STATISTICAL ANALYSIS

Data are expressed as mean ±standard deviation (SD) from triplicate recordings. Statistical processing was carried out using SPSS and Graph Pad Prism 6 software. The unpaired Student's t-test was used to compare date between groups that were normally distributed. P values less than 0.05 were considered statistically significant.

RESULTS

Low expression of lncRNA TUBA4B was found in non-small cell lung cancer

The expression of TUBA4B in 49 cases of NSCLC tumor tissues and their precancerous tissues was detected using RT-qPCR. The results manifested that the expression of TUBA4B in NSCLC tissues was apparently downregulated compared with that in the paracancerous tissues ($p<0.05$) (Figure 1A). The relationship between TUBA4B expression and clinicopathological characteristics of patients with non-small cell lung cancer was further analyzed. It was found that the expression level of TUBA4B was independent of the patient's gender and age (all $P>0.05$), but related to tumor size, lymph node metastasis, and TNM stage (all $p<0.05$) (Table 1). The expression of TUBA4B in human NSCLC cell lines (A549, PC9 and HCC827) and a normal human

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bronchial epithelial cell line HBE were also detected by RT-qPCR. The results showed that compared with HEB cells, the expression of TUBA4B in A549 ($p<0.01$), PC9 ($p<0.05$) and HCC827 ($p<0.05$) was reduced to varying degrees, and TUBA4B was significantly reduced in the A549 cell line (Figure 1B). Therefore, we choose A549 for subsequent experiments.

Table 1: TUBA4B expression and clinic pathological features in 49 non-small cell lung cancers.

Clinicopathological	Cases(n=49)	TUBA4B expression		p-value*
features		23 High(%)	26 Low(%)	
Gender				
Male	24	12(50.0)	12(50.0)	0.376
Female	25	11(44.0)	14(56.0)	
Age (years)				
≤60	23	11(47.8)	12(52.2)	0.742
>60	26	12(46.2)	14(53.8)	
Tumor size (mm)				
≤5.0	26	15(57.7)	11(42.3)	0.044*
>5.0	23	8(34.8)	15(65.2)	
TNM stage				
I-II	25	15(60.0)	10(40.0)	0.036*
III-IV	24	8(33.3)	14(66.7)	
Local invasion				
T1-T2	25	14(65.4)	11(34.6)	0.068
T3-T4	24	9(39.1)	15(60.9)	
Lymph-node metastasis				
0-2	27	16(59.3)	11(40.7)	0.031*
>2	22	7(31.8)	15(68.2)	
Histology				0.341
Adenocarcinoma	32	17(53.1)	15(46.9)	
Squamous	17	6(35.3)	11(64.7)	
*P values are calculated with Chi-square test.				

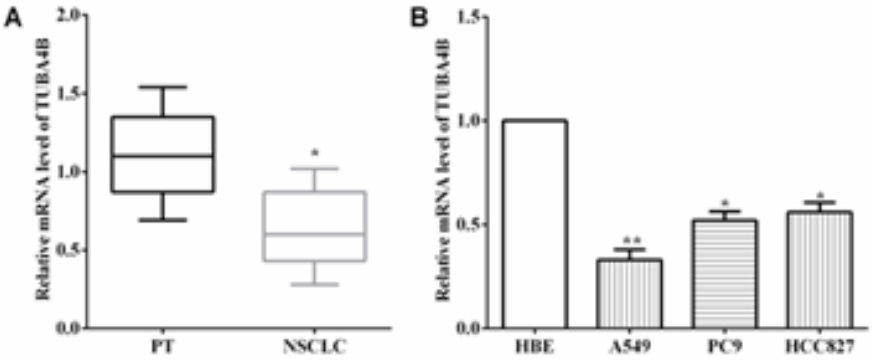

Figure 1: Low expression of lncRNA TUBA4B was found in non-small cell lung cancer (A) The expression of TUBA4B in tissues was apparently downregulated compared with that in the paracancerous tissues. (B) The expression of TUBA4B in NSCLC cell lines was lower than that of HEB cells.

TUBA4B suppressed the migration and invasion of A549 cells

The biological function of lncRNA TUBA4B is determined through the gains and losses of functional experiments. To further clarify the potential function of NEAT1, a TUBA4B overexpression plasmid (pcDNA-TUBA4B) was synthesized, and then transfected into A549 cells, and its transfection efficiency was checked by RT-qPCR. As expected, pGL4-TUBA4B increased the expression level of TUBA4B in A549 cells ($p<0.05$) (Figure 2A). In transwell experiments, overexpression of TUBA4B inhibited cell invasion ($p<0.05$) and migration ($p<0.05$) ability of non-small cell lung cancer. Overall, the findings indicate that TUBA4B regulates the migration and invasion of non-small cell lung cancer cells in vitro (Figure 2B).

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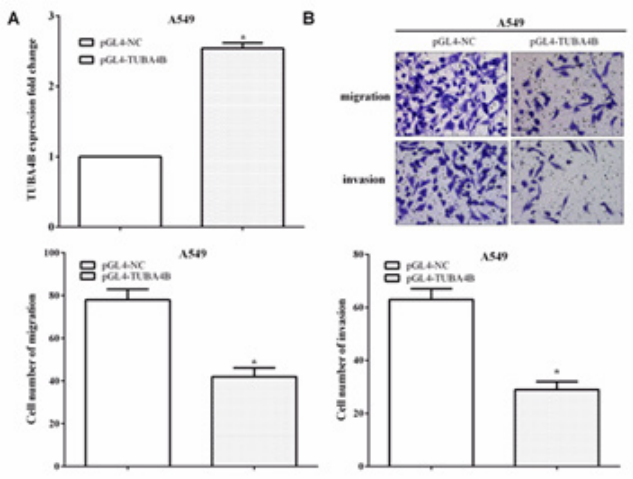

Figure 2: TUBA4B suppressed the migration and invasion of A549 cells (A) The transfection efficiency of transfecting was pGL4-TUBA4B in A549 cells was checked using RT-qPCR. (B) Overexpression of TUBA4B inhibited cell invasion and migration ability of A549 cells.

TUBA4B regulated the expression of miR-127 in A549 cells

We were pleasantly surprised to find that overexpression of TUBA4B regulated the expression of miR-127 ($p<0.05$) (Figure 3A). The expression of miR-127 in cell lines and tissue samples were evaluated using RT-qPCR. Not unfortunately, miR-127 expression was higher in human NSCLC cell lines A549 ($p<0.01$), PC9 ($p<0.05$) and HCC827 ($p<0.05$) than normal human bronchial epithelial cell line HBE (Figure 3B). Similarly, upregulation of miR-127 was also found in NSCLC tissues compared to corresponding paracancerous tissues ($p<0.05$) (Figure 3C). In addition, the expression of TUBA4B was positively correlated with the expression of miR-127 in NSCLC tissues ($p<0.05$) (Figure 3D).

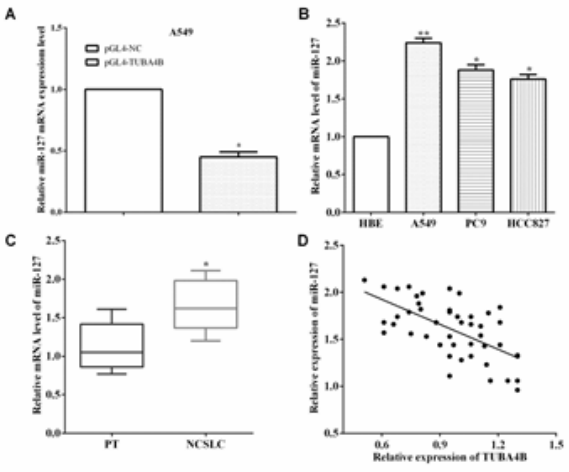

Figure 3: TUBA4B regulated the expression of miR-127 in A549 cells (A) Overexpression of TUBA4B regulated the expression of miR-127. (B) miR-127 expression was higher in human NSCLC cell lines than normal human bronchial epithelial cell line. (C) Upregulation of miR-127 was also found in NSCLC tissues compared to corresponding paracancerous tissues. (D) The expression of TUBA4B was positively correlated with the expression of miR-127 in NSCLC tissues..

Knockdown of miR-127 inhibited cell migration and invasion in A549 cells

In order to confirm the function of miR-127, we transfected the miR-127 inhibitor or inhibitor NC sequence into A549 cells, and determined the expression of miR-127 by qPCR. The results showed that miR-127 inhibitor significantly reduced miR-127 expression in these cells ($p<0.05$) (Figure 4A). The functional analyses showed that miR-127 inhibitor-transfected cells significantly reduced cell migration and invasion compared to NC-transfected cells (Figure 4B). All the results revealed that knockdown of miR-127 suppressed A549 cell migration and invasion.

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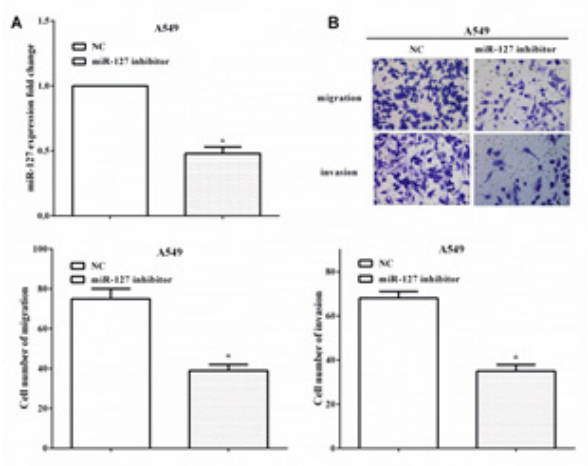

Figure 4: Knockdown of miR-127 inhibited cell migration and invasion in A549 cells (A) miR-127 inhibitor significantly reduced miR-127 expression in these cells. (B) Knockdown of miR-127 suppressed A549 cell migration and invasion.

miR-127 partly reverses the effects caused by overexpression of TUBA4B in vitro

In order to further study whether the tumor suppressive effect of TUBA4B is mediated by miR-127, TUBA4B or miR-127 mimics were co-transfected into A549 cells, and the transfection efficiency was detected by RT-qPCR ($p<0.05$) (Figure 5A). The results showed that after miR-124 was up-regulated, the cell invasion ($p<0.05$) and migration ($p<0.05$) ability increased (Figure 5B), indicating that miR-127 mimic greatly reduced the tumor suppressive effect of TUBA4B. All these data indicate that miR-127 can partially eliminate the inhibitory effect of TUBA4B on A549 cell invasion and migration.

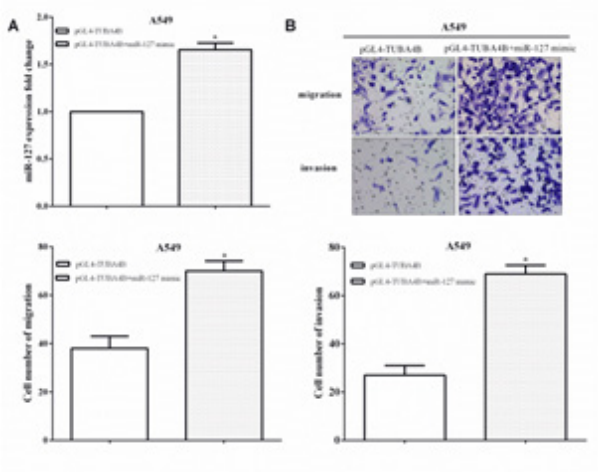

Figure 3: miR-127 partly reverses the effects caused by overexpression of TUBA4B in vitro (A) The transfection efficiency of co-transfected with TUBA4B or miR-127 mimic was calculated by RT-qPCR. (B) miR-127 mimic greatly reduced the tumor suppressive effect of TUBA4B on cell invasion and migration..

DISCUSSION

In past few decades, lung cancer is the most common malignancy and the most common cause of cancer deaths worldwide [21,22]. The morbidity and mortality of non-small cell lung cancer in China are still high, at 48.32/100,000 and 39.27/100,000 respectively [23]. Several reports have demonstrated that lncRNAs and miRNAs were closely linked to NSCLC. Our study investigated the function and potential molecular mechanisms of TUBA4B in non-small cell lung cancer. Our results demonstrate that TUBA4B expression is reduced in NSCLC tissues as well as NSCLC cell lines. Downregulation of TUBA4B inhibited the migration and invasion of A549 cells. In addition, TUBA4B negatively affects the expression of miR-127, and the expression of TUBA4B is negatively correlated with miR-127. TUBA4B inhibits the malignant behavior of A549 cells through the miR-127 pathway.

Long non-coding RNAs are eukaryotic genome with over 200 nucleotides in length, can activate or inhibit gene expression and thus participate in various biological processes [24,25]. Increasing evidence suggests that abnormally expressed lncRNA may involve in tumorigenesis. TUBA4B was found to be low expressed in breast cancer, and inhibited cell proliferation and invasion via directly targeting miR-9 [26]. In epithelial ovarian cancer, TUBA4B, associated with poor prognosis, inhibited cell proliferation and migration [26]. Previous evidence showed that TUBA4B was a poor predictor prognosis and it could regulate cell proliferation

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in non-small cell lung cancer [27]. However, the effect of TUBA4B on the metastasis of NSCLC is unclear. In this study, we found that TUBA4B was downregulated in NSCLC tissues and cell lines. Overexpression of TUBA4B impaired NSCLC cell migration and invasion. The expression of TUBA4B in NSCLC tissues was related to tumor size, TNM stage and lymph node metastasis. Although there is no obvious statistical correlation between the expression of TUBA4B and distant metastasis, it may be due to the small sample size. We will increase the sample size next.

Previous evidence indicated that lncRNAs could serve as a competitive endogenous RNA (ceRNA) to sponge miRNAs [28]. miRNAs may functioned as oncogenes in non-small cell lung cancer, including miR-942, miR-203, miR-122 and miR-539 [29-32]. miR-127 enhanced cell migration and invasion via targeting SEPT7 in glioblastoma [33]. Even in lung cancer, miR-127 promoted EMT and stem-like traits through a feed-forward regulatory loop [34]. Our findings uncovered that miR-127 was overexpressed in NSCLC tissues, and the expression of miR-127 has a negative connection with TUBA4B in NSCLC tissues. Moreover, miR-127 was upregulated in NSCLC cell lines in comparison with normal bronchial epithelial cell line. miR-127 expression was inhibited by exogenous overexpressed TUBA4B in A549 cells. Consistent with all the previous study, we discovered that knockdown of miR-127 inhibited A549 cell migration and invasion. In addition, miR-127 partially reversed partial suppressive roles of TUBA4B in NSCLC.

CONCLUSION

This study provides some new insights into the ceRNA mechanism regulated by TUBA4B in non-small cell lung cancer metastasis, and identified TUBA4B as a novel prognostic biomarker for the risk of metastasis. However, our study has a limitation that how TUBA4B regulated the expression of miR-127 in A549 cells is unclear, which we will research in next paper.

DECLARATIONS

Ethics approval and consent to participate

Ethics Committee of The first affiliated hospital ofShandong first medical university approved the research, and written informed consent was given by all participants.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIAL

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

COMPETING INTERESTS

All authors declare that they have no financial or other conflicts of interest in relation to this research and its publication.

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AUTHORS' CONTRIBUTIONS

Hongxi Guo as the corresponding author contributed to the conception of the study and performed the analysis; Xiyan Wang as the first author contributed significantly to write the manuscript and helped perform the analysis. All authors read and approved the final manuscript.

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