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ORIGINAL RESEARCH

RETRACTED ARTICLE: miR-765 Acts as a Tumor Promoter and Indicates Poor Prognosis in Non-Small Cell Lung Cancer

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Purpose: Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-related death worldwide with poor prognosis. Accumulating evidence indicates that miR-765 is an important regulator in the progression and prognosis of various cancers. In this study, the function in the progression and prognosis of NSCLC was investigated.

Patients and Methods: The expression of miR-765 in NSCLC was analyzed by qRT-PCR. The effect of miR-765 on cell proliferation, migration, and invasion of NSCLC was evaluated by CCK8 and Transwell assay. Kaplan–Meier analysis and Cox regression analysis were employed to assess the prognostic value of miR-765.

Results: The results demonstrated the significant upregulation of miR-765 in NSCLC tissues and cell lines relative to normal tissues and cells. High miR-765 expression was significantly correlated with the TNM stage of patients. Patients with high miR-765 expression showed a poorer prognosis than that of patients with low miR-765 expression. Cox analysis indicated that miR-765 could be considered as an independent prognostic factor for NSCLC. Additionally, the upregulation of miR-765 was revealed to promote NSCLC cell proliferation, migration, and invasion by targeting BMP6.

Conclusion: The overexpression of miR-765 in NSCLC was associated with TNM stage and poor prognosis of patients. miR-765 served as a tumor promoter of NSCLC by regulating BMP6. These findings provide a potential biomarker and therapeutic target for the prognosis and treatment of NSCLC.

Keywords: non-small cell lung cancer, miR-765, prognosis, progression

Introduction

Lung cancer is a malignant tumor with the highest morbidity and mortality worldwide.[1] Non-small cell lung cancer (NSCLC) is one of the most common pathological types of lung cancer that comprises a large number of lung cancer cases. NSCLC is the leading cause of cancer-related death due to its poor prognosis.[2] Uncontrolled cell growth and invasive metastasis of NSCLC result in the high morbidity and mortality.[3] The lack of effective screening tests and therapy is the main cause of the poor survival rate of NSCLC patients. Currently, the recognition of differential prognostic markers has drawn special attention and the identification of useful indicators for accurate prediction of clinical outcomes is essential to improve the prognosis of NSCLC.[4]

Biomarkers can help detect the presence and development of various human diseases.[5] Recently, microRNAs (miRNAs) have been identified as efficient and accurate biomarkers in various cancers. miRNAs are a class of small non-coding RNA with a length from 18 to 24 nucleotides, which widely exists in both higher and

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lower organisms and function as post-transcriptional regulators.[6,7] Numerous researches have shown that miRNAs are associated with the carcinogenesis and progression of diverse human cancers, such as breast cancer, cervical cancer, prostate cancer, and gastric cancer.[8–11] A number of miRNAs have been identified as the potential biomarkers for NSCLC due to the involvement in tumor progression. For example, miR-503 could suppress the progression of NSCLC and regulates the resistance of NSCLC cells to cisplatin.[12,13] Increasing evidence confirms the close relationship between miR-765 and various types of human cancers. In hepatocellular carcinoma, miR-765 was upregulated and promoted the proliferation and tumorigenicity by targeting INPP4B.[14] It also has been reported that miR-765 was dysregulated and participated in the progression of many other cancers, including esophageal squamous cell carcinoma, breast cancer, prostate cancer, and renal cell carcinoma, etc.[15–18]

In the previously reported miRNA expression file in NSCLC, miR-765 was found to be upregulated but its role in the progression of NSCLC was unclear. This study focused on the role of miR-765 in the prognosis and progression of NSCLC by qRT-PCR, CCK8, and Transwell assay aimed to provide a novel insight into the therapy and management of NSCLC.

Patients and Methods

Tissues and Cell Lines

This study was approved by the Ethics Committee of Shanghai Pulmonary Hospital and in accordance with the Declaration of Helsinki. 126 paired tissues were collected from NSCLC patients who had undergone surgery at Shanghai Pulmonary Hospital during 2012–2014. Patients all have signed informed consent. The survival information of participants was obtained by a 5-year follow-up survey by telephone 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, and 60 months after the surgery. Tumor tissues and para-carcinoma normal tissues were frozen in liquid nitrogen and stored at −80°C until use.

A549, NCI-H1299, NCI-H1648, and HCC827 were chosen as NSCLC cell lines with BEAS-2B as a normal lung epithelial cell. All cell lines were purchased from ATCC and cultured in RPMI1640 medium with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO_2.

Cell Transfection

Cells were transfected with miR-765 mimic, miR-765 inhibitor, miR-765 mimic NC, or miR-765 inhibitor NC with the Lipofectamine 2000 Reagent (Invitrogen, USA) according to the instructions of the manufacturer, to regulate the expression level of miR-765. The transfection efficiency was evaluated by detecting the expression of miR-765 in transfected cells.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from frozen tissues and cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from total RNA with a Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.). qRT-PCR was performed with SYBR Green I Master Mix kit (Invitrogen) and the 7300 Real-Time PCR System (Applied Biosystems, USA). We employed the $2^{-\Delta\Delta Ct}$ method to calculate the relative expression of miR-765 with U6 as the normalization calibrator. The sequences of miR-765 primer and U6 were as follows: forward 5′-GGCTCGGATCCGTTAG-3′ and reverse 5′-CGACTACCGTTAGCTAGA-3′ for miR-765 primer; forward 5′-CGCTTCGGCAGGCATTATATAC-3′ and reverse 5′-AGGGGCCATGCTAATCTT-3′ for U6.

Cell Proliferation Assay

Cell proliferation was detected by the CCK8 assay. Briefly, cells were inoculated into 96-well plates with a cell density of 5×10^3 cells per well incubating with the culture medium. Then, 10 μL CCK8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added to each well after 0, 1, 2, and 3 days and continued incubating for 4 h at 37°C with 5% CO_2. Finally, the absorbance of each well at 450 nm was measured with the help of a microplate reader (Thermo Fisher Scientific).

Transwell Migration and Invasion Assay

Transwell migration and invasion assay was conducted in 24-well transwell chambers (8-μm pore size; Multiskan MK3, Thermo, Waltham, MA, USA). Cells were seeded into the upper chamber of transwell plates with a cell density of 1×10^5 cells per well and cultured with serum-free medium. For invasion assay, the upper chamber was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) before cells seeding. Medium containing 10% FBS was added to the bottom chamber as a chemoattractant. After 48 h of culture, cells were fixed with methanol and stained with 0.1% crystal violet. The number of migrated and invasive cells was counted by a light microscope.

Dual-Luciferase Reporter Assay

The potential targets of miR-765 were predicted with online software (http://www.targetscan.org) and validated with the dual-luciferase reporter assay. The 3′UTR region sequence of BMP6 was amplified by genomic DNA and inserted into the pGL3 luciferase reporter vector. Nucleotides in the predicated miR-765 binding region were cloned into the pGL3 reporter vector and also mutated. Constructed reporter plasmids and miR-765 mimics, inhibitor, or negative controls were co-transfected into A549 with the Lipofectamine 2000 reagent. The relative luciferase activity of BMP6 was analyzed with the Dual-Luciferase assay kit (Promega Corporation), after 24 h of the transfection.

Statistical Analysis

Statistical analysis was performed with SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0 software (GraphPad Software, Inc., Chicago, USA). The values are expressed as the mean ± standard deviation (SD). Differences between groups were analyzed by Student's *t*-test and one-way ANOVA. Correlation analysis was performed by the χ^2 test and Cox regression analysis. Survival analysis was conducted by Kaplan-Meier analysis. Differences were considered statistically significant when $P < 0.05$.

Results

Comparison of miR-765 Expression Level in NSCLC and Normal Tissues

The expression of miR-765 in 12 pairs of NSCLC and para-carcinoma normal tissues was measured by qRT-PCR analysis (The delta CT data were summarized in Supplementary Table 1). Compared with para-carcinoma normal tissues, miR-765 was significantly upregulated in NSCLC tissues ($P < 0.001$, Figure 1A). Similar results in cell experiments, the significant upregulation of miR-765 was found in NSCLC cell lines (A549, HCC827, NCI-H1648, HCI-H1299) in comparison with normal cell line BEAS-2B ($P < 0.001$, Figure 1B).

Relationship Between miR-765 Expression Level and Clinicopathological Parameters of NSCLC Patients

The potential correlation between miR-765 expression level and the clinical features of NSCLC patients was assessed by the χ^2 test, which was summarized in Table 1. The expression of miR-765 was considered as either high ($n = 69$) or low ($n = 57$) in NSCLC tissues according to the cut-off value, which was defined as the average of the cohort. There was a significant relationship between the expression level of miR-765 and TNM stage of patients ($P = 0.019$), but no significant association was found between miR-765 expression level and other clinical factors, including age, gender, tumor size, differentiation, histological subtypes, and lymph node metastasis ($P > 0.05$, Table 1).

Relationship Between miR-765 Expression Level and Clinical Prognosis of NSCLC Patients

Follow-up data were collected from recruited patients during a period of 5 years. The Kaplan-Meier survival analysis

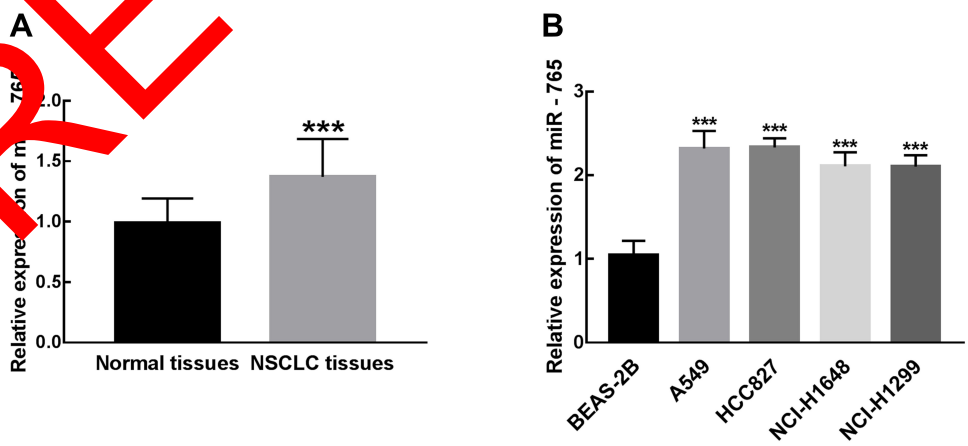

Figure 1 Differential expression of miR-765 in NSCLC tissues and cell lines compared with normal tissues and cells. (**A**) miR-765 expression was significantly increased in NSCLC tissues relative to para-carcinoma normal tissues (***$P < 0.001$). (**B**) The expression of miR-765 in NSCLC cell lines (A549, HCC827, NCI-H1648, HCI-H1299) was significantly higher than that in normal cell line BEAS-2B (***$P < 0.001$).

Table 1 The Relationship Between Clinicopathological Parameters of Patients and miR-765 Expression in NSCLC Tissues

	Total Patients (n = 126)	Expression of miR-765		*P* value
		High (n = 69)	Low (n = 57)	
Age				0.682
≤ 60	65	33	32	
> 60	61	36	25	
Gender				0.548
Male	70	37	33	
Female	56	32	24	
Tumor size (cm)				0.128
≤ 4	68	35	33	
> 4	58	34	24	
Differentiation				0.18
Well + moderate	83	36	47	
Poor	43	33	1	
Histological subtypes				0.496
Squamous-cell carcinoma	38	17	21	
Adenocarcinoma	54	31	23	
Large cell carcinoma	24	15		
Others	10	6	4	
Lymph node metastasis				0.138
Negative	75	37	38	
Positive	51	32	19	
TNM stage				0.019
I–II	86	45	41	
III–IV	40		16	

was performed to evaluate the prognostic value of miR-765 in NSCLC patients. With the help of the Log rank test, it was found that the survival rate of NSCLC patients with a high expression of miR-765 was lower than that of patients with low miR-765 expression level (Log rank test $P = 0.012$, Figure 2). Notably, multivariable Cox regression analysis demonstrated the close association between miR-765 expression (HR = 2.226, 95% CI = 1.098–4.513, $P = 0.027$) and the survival rate of NSCLC patients, which indicated that miR-765 expression was an independent factor predicting the 3-year survival rate of NSCLC patients. Additionally, the TNM stage of patients was also identified as an independent prognostic factor of NSCLC patients (HR = 1.991, 95% CI = 1.065–3.722, $P = 0.031$ Table 2).

miR-765 Promotes NSCLC Cell Proliferation

A549 and HCC827 were transfected with miR-765 mimic or miR-765 inhibitor and the expression of miR-765 after the transfection was confirmed by qRT-PCR. The significant overexpression and knockdown of miR-765 were found in A549 and HCC827 after the transfection with miR-765 mimic and inhibitor, respectively ($P < 0.001$, Figure 3A).

The CCK8 assay was used to assess the effect of miR-765 expression on NSCLC cell proliferation. The results showed that the knockdown of miR-765 dramatically suppressed the proliferation of NSCLC cells compared with blank control ($P < 0.05$, Figure 3B). While the overexpression of miR-765 by the transfection of miR-765 mimic significantly promoted the proliferation of NSCLC cells ($P < 0.05$, Figure 3B). Two corresponding negative control (mimic NC and inhibitor NC) showed no significant difference with the blank control ($P > 0.05$).

miR-765 Promotes NSCLC Cell Migration and Invasion

The Transwell assay was used to evaluate the effect of miR-765 expression on cell migration and invasion of

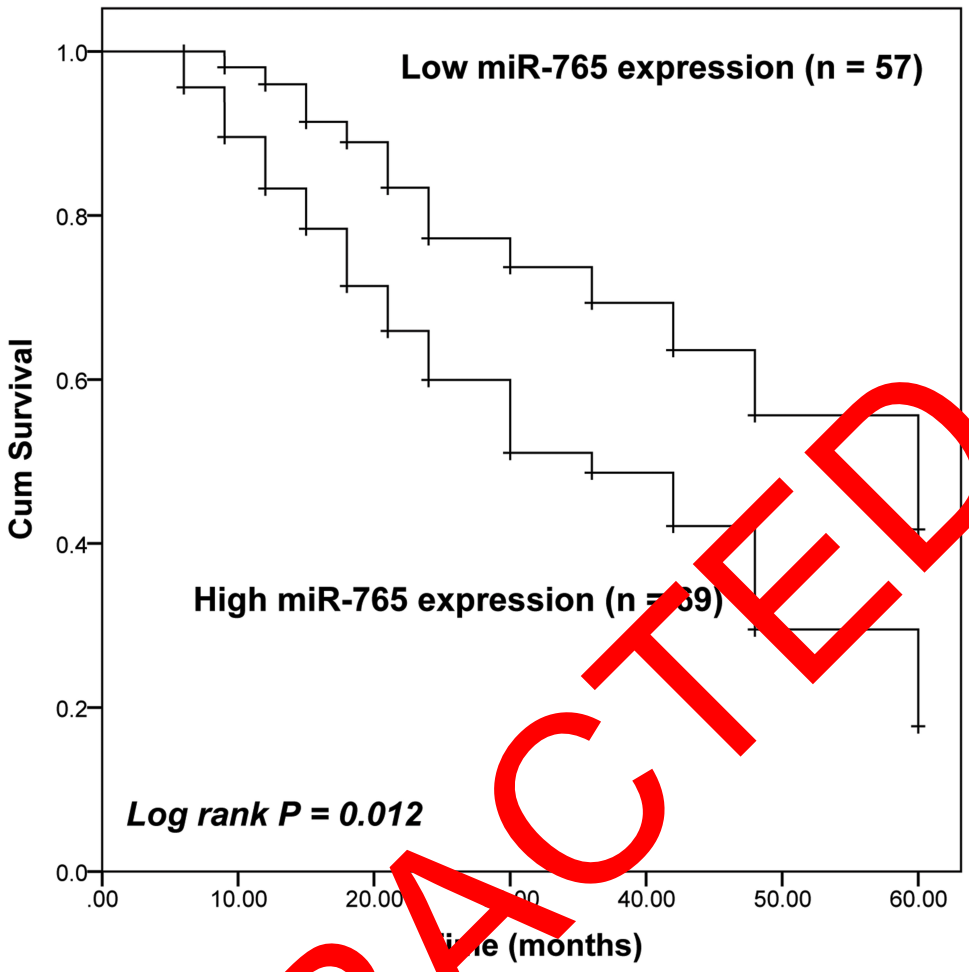

Figure 2 Kaplan-Meier survival curves of the whole cohort of 126 NSCLC patients with different miR-765 expression. Patients with high miR-765 expression had a poorer prognosis than that of patients with low miR-765 (Log rank $P = 0.012$).

NSCLC. miR-765 overexpression induced a significant increase in the number of migrated and invasive cells of A549 and HCC827 relative to blank control, whereas these processes were significantly inhibited by the knockdown of miR-765 ($P < 0.01$, Figure 4A and B).

Table 2 Multivariable Analysis of NSCLC Survival by Cox Proportional Hazards Model

	HR Factor	95% CI	*P* value
miR-765	2.226	1.098–4.513	0.027
Age	1.156	0.642–2.080	0.630
Gender	1.522	0.792–2.926	0.207
Tumor size	1.611	0.851–3.051	0.143
Differentiation	1.619	0.832–3.152	0.156
Histological subtype	1.439	0.452–4.577	0.322
Lymph node metastasis	1.501	0.813–2.771	0.195
TNM stage	1.991	1.065–3.722	0.031

BMP6 Serves as a Direct Target of miR-765

The potential targets of miR-765 was predicted with the help of online software (Supplementary Table 2). The direct relationship between BMP6 and miR-765 was estimated by the dual-luciferase reporter assay. The binding sites between miR-765 and the 3′UTR of BMP6 were predicted by the online software and shown in Figure 5A. Co-transfection of miR-765 mimic and BMP6 WT vector significantly inhibited the relative luciferase activity of BMP6, which was dramatically enhanced by the transfection of miR-765 inhibitor ($P < 0.001$, Figure 5B). The luciferase activity of BMP6 MT was not influenced by the dysregulation of miR-765 ($P > 0.05$, Figure 5B).

Discussion

In contrast to the steadily improving survival rate of most cancers, the 5-year survival rate of NSCLC was still lower

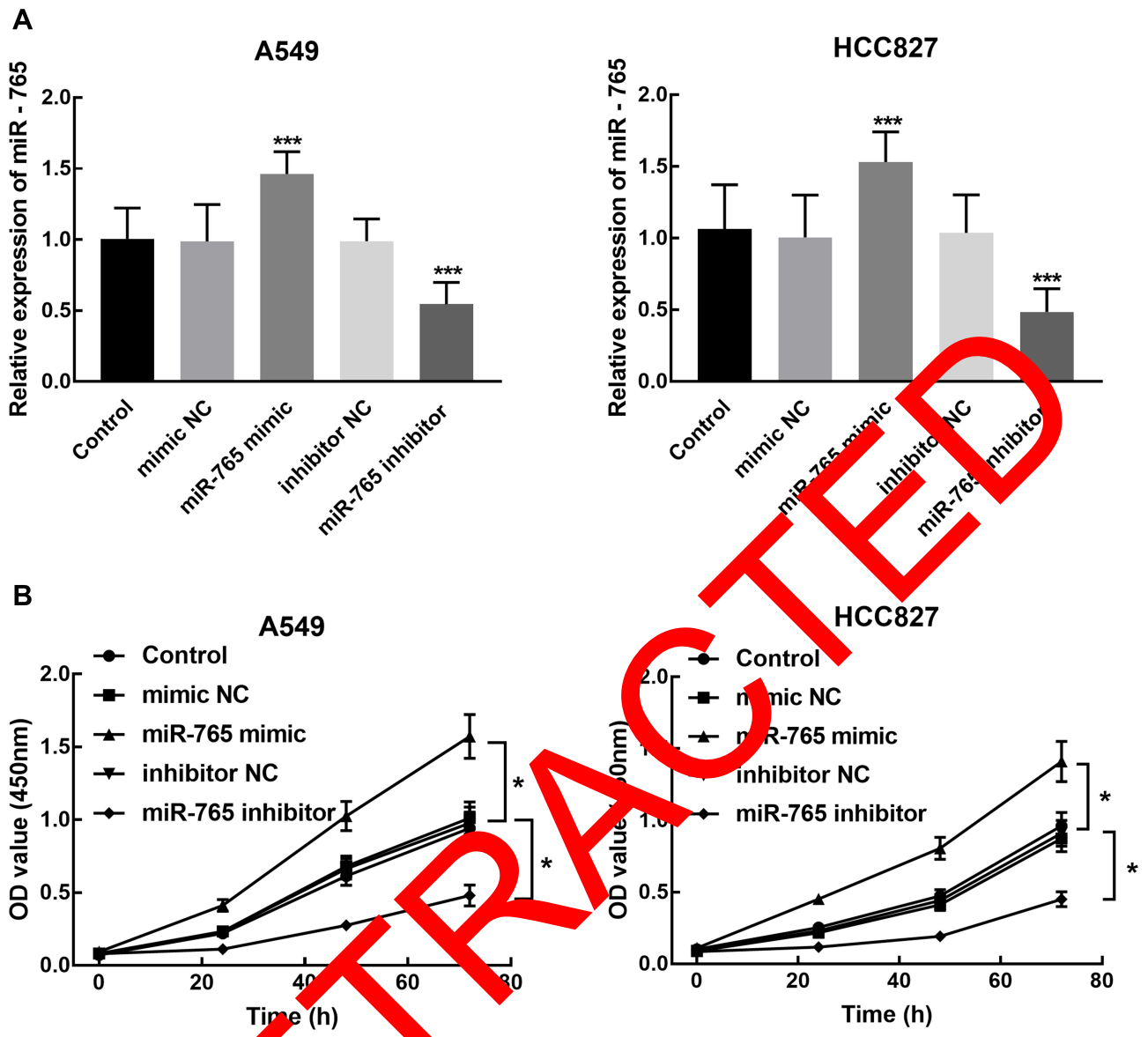

Figure 3 Cell transfection efficiency and its effect on NSCLC cell proliferation. (**A**) miR-765 expression was significantly increased by miR-765 mimic transfection and inhibited by miR-765 inhibitor transfection in A549 and HCC827 (***$P < 0.001$). (**B**) CCK8 assay showed the inhibition of A549 and HCC827 proliferation by the transfection of miR-765 inhibitor and the promotion by the transfection of miR-765 mimic (*$P < 0.05$).

than that of other cancers.[19] Numerous genetic factors including dysregulated miRNAs have been reported to be involved in the pathogenesis, development, metastasis, and prognosis of various cancers. For example, miR-1231 inhibited cell proliferation, migration, and invasion of prostate cancer by targeting EGFR.[20] miR-888 acts as an oncogene in colorectal cancer and is associated with the poor prognosis of patients.[21] Many miRNAs have been associated with tumor-suppressive or promoted effects in NSCLC. miR-3607 has been shown to be downregulated in NSCLC and was considered as an independent predictor for overall survival of patients, which indicated miR-3607 could be a novel and stable biomarker for NSCLC.[22] miR-4317 could inhibit the progression of NSCLC by targeting Fibroblast growth factor 9 and Cyclin D2.[23] miR-765 was identified as one of the upregulated miRNAs in the miRNA expression profile of NSCLC, which makes it possible to participate in the progression and prognosis of NSCLC.[24]

Previously, changes in the expression of miR-765 have been demonstrated to play vital roles in regulating the progression of different types of cancers. miR-765 is downregulated and inhibits the migration of oral squamous cancer cells by targeting EMP3.[25] It also reported miR-765

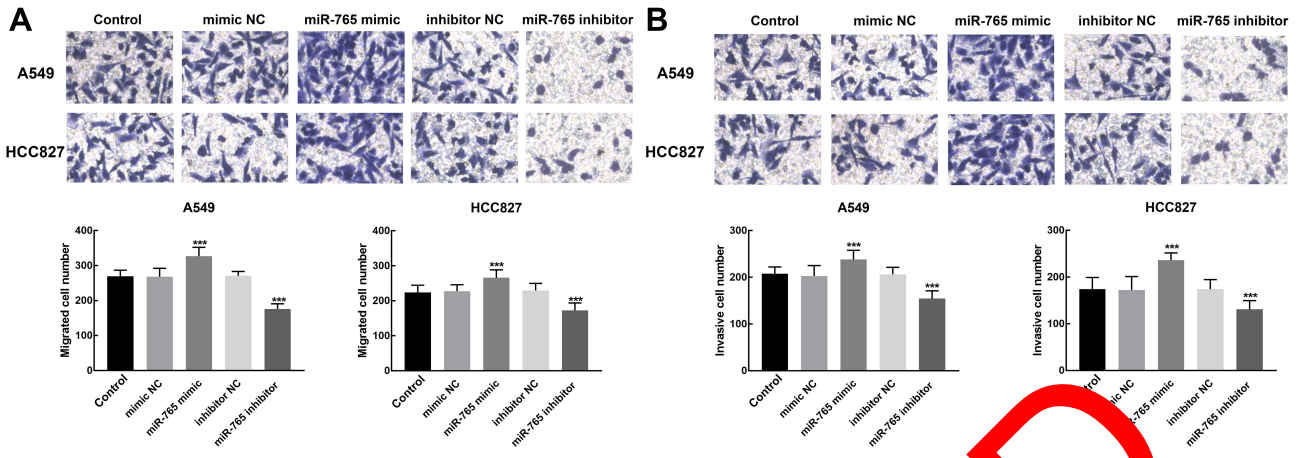

Figure 4 Effect of miR-765 expression on NSCLC cell migration and invasion. (**A**) The migration of A549 and HCC827 was inhibited by the knockdown of miR-765 and promoted by the overexpression of miR-765 (***$P < 0.001$). (**B**) The invasion of A549 and HCC827 was inhibited by the knockdown of miR-765 and promoted by the overexpression of miR-765 (***$P < 0.001$).

regulated EMPS to inhibit breast carcinoma.[26] The expression of miR-765 was different in different cancers. It was reported to be upregulated in multiple myeloma and served as a potential therapeutic target of multiple myeloma prevention and treatment.[27]

Here, we found the upregulation of miR-765 in NSCLC, which is associated with the TNM stage of patients. Upregulation of miR-765 played a tumor promoter role in NSCLC, because of its promoted effects on cell proliferation, migration, and invasion of NSCLC. Differences in miRNA expression in tumor samples could exert different effects on the function of tumor cells. The downregulation of miR-765 by the transfection of miR765 inhibitor had the opposite effects, which inhibited the proliferation, migration, and invasion of NSCLC cells. Additionally, miR-765 was also found to be associated with the prognosis of NSCLC patients and could be considered as an independent prognostic indicator for NSCLC. Previously, some studies have reported the prognostic value of miR-765 in many other cancers and tumors. In osteosarcoma (OS), miR-765 could sensitize OS cells to cisplatin by downregulating APE1 and was associated with the prognosis of OS patients.[28] Moreover, miR-765 could also promote the progression of OS via MTUS1/ERK/EMT axis.[29] Upregulation of miR-765 predicates a poor prognosis of esophageal squamous cell carcinoma.[15] These results indicated the potential involvement of miR-765 in the progression of NSCLC and served as a potential biomarker for the prognosis of NSCLC.

Although the mechanism by which miRNA alters gene expression remains controversial, it is also necessary to ascertain the mechanism underlying the function of miR-765 in NSCLC. Previously, the role of miR-765 in OS and hepatocellular carcinoma has been demonstrated to result from the regulation of MTUS1 and INPP4B by miR-765.[14,29] BMP6 is one of the major members of the transforming growth factor-β superfamily, which is involved in the growth and development of normal tissues and various tumors. Previously, BMP6 was considered as a tumor suppressor of NSCLC, which was also associated with the poor prognosis of patients. Here, the relative luciferase activity of BMP6 was found to be inhibited by the overexpression of miR-765 and enhanced by the knockdown of miR-765, indicating that miR-765 promoted cell proliferation, migration, and invasion of NSCLC by targeting BMP6. SMAD and ERK1/2 signaling were reported to be involved in the regulating follicular function of BMP-6 in human granulosa cells.[30] Therefore, it was speculated that the regulation of BMP6 by miR-765 in NSCLC may be through these two signalings.

However, there is still a limitation of this study. The treatment after surgery of patients is a vital factor that might affect the prognosis of patients. In this study, the treatment of patients was not defied, which may influence the results.

Conclusion

In conclusion, the results of this study suggest that miR-765 is upregulated in NSCLC and its upregulation indicates a poor prognosis of patients. It is also suggested that the proliferation, migration, and invasion of NSCLC cells were promoted by the upregulation of miR-765 and inhibited by the downregulation of miR-765. These findings

A

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context+ score
Position 280-286 of BMP6 3' UTR hsa-miR-765	5' ...GAAGCUCUUCCUACCCUCCUCCC... 3' GUAGUGGAAGGAA--GAGGAGGU	7mer-m8	-0.22
Position 638-644 of BMP6 3' UTR hsa-miR-765	5' ...AUUUCUACACCUCAA---UCCUCCAU... 3' GUAGUGGAAGGAAGAGGAGGU	7mer-A1	-0.19

B

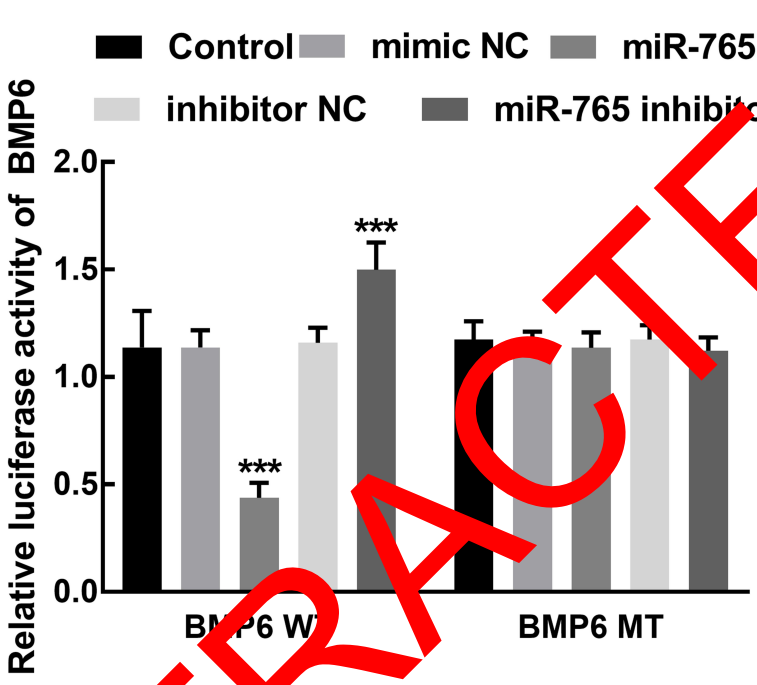

Figure 5 The target prediction of miR-765. (**A**) The binding sites between miR-765 and the 3'UTR of BMP6. (**B**) The overexpression of miR-765 significantly inhibited the relative luciferase activity of BMP6, while the knockdown of miR-765 dramatically enhanced the relative luciferase activity of BMP6. ***$P < 0.001$.

indicated the potential biomarker role of miR-765 in the prognosis and progression of NSCLC, which provides a novel potential therapeutic target for NSCLC.

Disclosure

The authors report no conflicts of interest in this work.

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