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JMJD3 enhances invasiveness and migratory capacity of non-small cell lung cancer cell *via* activating EMT signaling pathway

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Abstract. – OBJECTIVE: This study aimed to explore the expression of JMJD3 in non-small cell lung cancer (NSCLC) and to further study its association with clinical features and prognosis of patients.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine the level of JMJD3 in 46 pairs of NSCLC tissues and para-cancerous specimens. The relationship between JMJD3 level and clinical features of NSCLC and patients' prognosis was analyzed. And JMJD3 expression in NSCLC cells was further verified by qRT-PCR. In addition, JMJD3 knockdown model was constructed using siRNA in cell lines including A549 and SPC-A1, and the effect of JMJD3 on the biological function of NSCLC cells was analyzed by cell counting kit-8 (CCK-8) and transwell migration and invasive-ness assays. Lastly, Western blot was performed to explore the potential mechanism.

RESULTS: In this investigation, qRT-PCR results indicated that JMJD3 expression in above-mentioned tumor tissues was conspicuously higher than that in normal tissues. In addition, compared with patients with low level of JMJD3, patients with high level of JMJD3 had a higher incidence of lymphatic metastasis and distant metastasis and a lower overall survival rate. Meanwhile, the proliferation, invasiveness and migratory capacity of cells in the sh-JMJD3 group was conspicuously decreased when compared with the cells in negative control group. Western Blot results indicated that the levels of key proteins in EMT signaling pathway such as E-cadherin, N-cadherin, Vimentin, TGF-β, and MMP-9 were notably decreased in sh-JMJD3 group. Besides, the addition of TGF-β cytokines synergistically promoted the malignant progression of NSCLC induced by JMJD3.

CONCLUSIONS: JMJD3 expression was found conspicuously increased in NSCLC, which might be close relevant to NSCLC lymphatic or distant metastasis as well as patients' poor prognosis. Therefore, we speculated that JMJD3 could promote invasiveness and migratory capacity of non-small cell lung cancer cells by activating EMT process.

Key Words:
JMJD3, EMT, Non-small cell lung cancer, Invasiveness and migratory capacity.

Introduction

Lung cancer is the most common malignant tumor in the world, and the mortality rate ranks first among malignant tumors, posing a great threat to the health and life of the population[1-3]. This cancer accounts for 17% of new cancer cases worldwide and 23% of new cancer deaths[1-4]. In China, along with social progress and economic development, environmental pollution has also been brought. Coupled with the change of population structure, the elderly population has been increasing, and the incidence of lung cancer has been increasing rapidly, creating a huge social and economic burden[5-7]. Risk factors for lung cancer include tobacco, environmental pollution, food, genetics, and chronic obstructive pulmonary disease. Lung cancer can be divided into small cell lung cancer and non-small cell lung cancer (NSCLC) according to histological type, the latter including squamous cell carcinoma, adenocarcinoma, and large cell carcinoma[4,7]. Compared with other malignant tumors, lung cancer has the characteristics of high incidence, high mortality, and difficulty of early diagnosis[8]. Cancer in early stage can be cured by surgical resection[9]. However, lung cancer often has no symptoms in the early stage, and it has been advanced or metastasized at the time of diagnosis, and thus lost the chance of surgery[9,10]. For patients who cannot be treated surgically, radiotherapy can also be used to improve clinical symptoms, elevate patients' quality of life, and prolong their survival rate[11,12]. Therefore, the key to improving the survival rate of patients with lung cancer is early detection, aggressive treatment, and timely intervention[12].

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Histone (H) is an important part of the chromosome. It not only exists as a support for DNA, but also plays an important role in gene level regulation and gene imprinting[13,14]. Histones have a variety of post-translational modifications, including acetylation, ubiquitination, and methylation, which greatly enrich the structure and function of histones and make histones become one of the research hotspots in epigenetics[14,15]. Among them, the research on methylation of histones is currently a very important research direction[15]. In recent years, many scholars[16,17] have shown that there is a close relationship between the abnormal methylation function of histones and the occurrence and development of tumors, and the process of such methylation modification is found to be reversible. In-depth study of the methylation of histones is not only benefit to researchers to understand a series of physiological mechanisms such as gene expression and gene regulation, but also to the judgement of diagnosis, prevention, and prognosis of some major diseases such as cancer[17,18]. A long time ago, researchers[18,19] found that acetylation and phosphorylation of histones are reversible, but since no histone demethylase has been found, histone methylation has been considered to be a stable and irreversible epigenetic modification process. JMJD3 (jumonji domain-containing protein 3) is a histone demethylase of H3K27 me2/me3, which is thought to be closely related to the development and immunity of nerves and epidermal cells, and it is also important for transcription and activation of chromatin[20-22].

Epithelial-mesenchymal transition (EMT) refers to the phenomenon that epithelial cells transform into mesenchymal cells under normal physiological and specific pathological conditions. The existing theory suggests that EMT may play an important role in regulating the malignant biological behavior of tumors[23-25]. In this study, we analyzed the level of JMJD3 in 46 pairs of NSCLC tissues and adjacent tissues and explored the effects of JMJD3 on the biological function of NSCLC cells. Previous reports have indicated that JMJD3 can promote the process of tumor cell invasion and metastasis, thus controlling the development of tumors. This work was designed to investigate the relationship between JMJD3 expression and pathological parameters, as well as prognosis of patients with non-small cell lung cancer and the specific mechanisms involved in cancer progression.

Patients and Methods

Patients and NSCLC Samples

We collected 46 pairs of tumors and paracancerous tissues from patients with NSCLC. According to the 8th edition of UICC/AJCC NSCLC TNM staging criteria, all patients were diagnosed as NSCLC by postoperative pathological analysis and did not receive anti-tumor treatment such as radiotherapy or chemotherapy before surgery. The study has been approved by the Ethics Oversight Committee. Patients and their families had been fully informed that their specimens would be used for scientific research and signed the informed consent.

Cell Lines and Reagents

Five human LCa cells (A549, H1299, PC-9, H358, SPC-A1) and one normal human bronchial epithelial cell (BEAS-2B) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), and Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). Cells were cultured in an incubator with 5% CO_2 at 37°C.

Transfection Experiment

Negative control (NC) sequence and siRNA (sh-JMJD3) containing the JMJD3 interference sequence were purchased from Shanghai Jima Company (Shanghai, China). Cells were seeded in 6-well plates and grown to a cell density of 70%; then, siRNA transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Cells were collected 48 h later for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay and function experiments.

Cell Counting Kit-8 (CCK-8) Proliferation Assay

The proliferation of lung cancer cells was detected using the CCK-8 kit (Dojindo, Kumamoto, Japan). The main steps were as follows. First, 100 uL of cell suspension was added in each well of the culture plate (including 2000 cells). After cell adherence finished, 10 uL of CCK-8 solution was added in each well and continued to incubate the cells for 1 h in the culture incubator. Lastly, cell proliferation situation was measured using a microplate reader at 450 nm of absorbance. Wells with the corresponding amount of cell culture medium and CCK-8 solution but no cells added were used as blank controls.

Transwell Cell Migration and Invasion Assay

The cells after transfection for 48 hours were collected, centrifuged and resuspended in medium without FBS to adjust the density to 5 x 10^5 cells/mL. 200 uL (1 x 10^5 cells) of cell suspension was added to the upper chamber, and 700 uL of a medium containing 20% FBS was added to the lower chamber. The cells were placed back in the incubator for a specific period of time depending on the migration capacity of each cell line. The transwell chamber was clipped, washed 3 times with 1 x PBS, and placed in methanol for 15 min for cell fixation. After the chamber was stained in 0.2% crystal violet for 20 min, the cells on the upper surface of the chamber were carefully wiped off with water and a cotton swab. The perforated cells stained in the outer layer of the basement membrane of the chamber were observed under the microscope, and 5 fields of view were randomly selected.

QRT-PCR

Total RNA was extracted from NSCLC cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was reverse transcribed into cDNA using Primescript RT Reagent. The qRT-PCR reaction was then carried out. The following primers were used: JMJD3: F: 5'-GCCCTCTGTGCTACTTACTC-3', R: 5'-GCTGGTTGTGGGTTACTCTC-3'; β-actin: F: 5'-CCTGGCACCCAGCACAAT-3', R: 5'-TGCCGTAGGTGTCCCTTTG-3'. Data analysis was performed using ABI Step One software, and relative mRNA levels were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Western Blot Assay

The transfected cells were lysed using cell lysis buffer, shaken on ice for 30 minutes, and centrifuged at 14,000 x g for 15 minutes at 4°C. The total protein concentration was calculated using bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). The extracted proteins were separated *via* a 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The Western blot detection was performed according to the standard procedures. The primary antibodies were E-cadherin, N-cadherin, Vimentin, TGF-β, MMP9, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the secondary antibodies were anti-mouse and anti-rabbit, all purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical Analysis

GraphPad Prism 5 V5.01 software (La Jolla, CA, USA) was applied to perform statistical analysis. The differences between the two groups were analyzed using the Student's *t*-test. The comparison between groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Independent experiments were repeated at least three times for each experiment and the measurement data were expressed as mean ± standard deviation. $p<0.05$ was considered statistically significant.

Results

JMJD3 was Highly Expressed in NSCLC Tissues and Cell Lines

To determine the role of JMJD3 in NSCLC, we collected 46 pairs of NSCLC tumor tissue specimens and para-cancerous ones and detected the difference of JMJD3 expression in above specimens using qRT-PCR. It was found that JMJD3 was elevated in NSCLC tissues compared with normal tissues (Figure 1A, B), suggesting that JMJD3 might play a role as a tumor-promoting gene in NSCLC. At the same time, we examined JMJD3 expression in commonly used NSCLC cell lines. Among them, JMJD3 was highly expressed in A549 and SPC-A1 cell lines (Figure 1C).

JMJD3 Level Was Correlated With Lymph Node and Distance Metastasis and Overall Survival Rate of NSCLC Patients

Specimens were divided into two groups based on JMJD3 expression in these tissues, including highly expressed group and lowly expressed one. Chi-square test was used to analyze the relationship between JMJD3 level and age, gender, clinical stage, lymph node or distant metastasis of NSCLC patients. Table I indicated that a high level of JMJD3 was positively related to the occurrence rate of metastasis, but not with age and gender. In addition, to figure out the association between JMJD3 expression and the prognosis of patients with NSCLC, we collected relevant follow-up data. Kaplan-Meier survival curves revealed that high level of JMJD3 was conspicuously associated with patients' poor prognosis, and the higher the level of JMJD3, the worse the prognosis ($p<0.05$; Figure 1D).

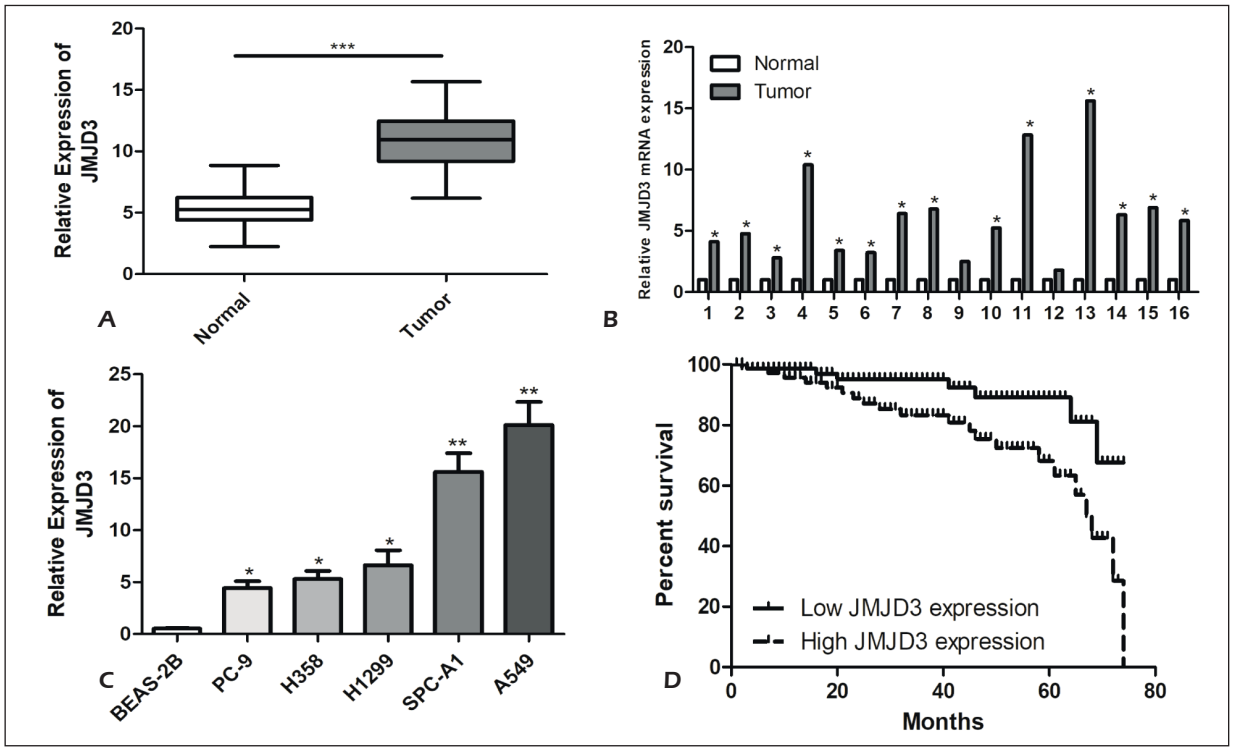

Figure 1. JMJD3 was highly expressed in non-small cell lung cancer tissues and cell lines. **A-B**, qRT-PCR detection of JMJD3 expression differences in non-small cell lung cancer tumor tissues and adjacent non-tumor tissues. **C**, qRT-PCR detection of JMJD3 expression levels in non-small cell lung cancer cell lines. **D**, Kaplan Meier survival curve revealed that the prognosis of patients with high expression of JMJD3 was significantly worse than that of low expression. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Knockdown of JMJD3 Inhibited Cell Migration Activity and Invasiveness

The JMJD3 interference model was constructed and the transfection efficiency was verified by qRT-PCR (Figure 2A and 2B). Subsequently, CCK-8 assay revealed that the proliferation of cells in sh-JMJD3 group was notably reduced compared with that in control group (Figure 2C).

Table I. Association of JMJD3 expression with clinicopathologic characteristics of lung cancer.

Parameters	No. of cases	JMJD3 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.417
<60	17	12	5	
≥60	29	17	12	
Gender				0.489
Male	22	15	7	
Female	24	14	10	
T stage				0.828
T1-T2	28	18	10	
T3-T4	18	11	7	
Lymph node metastasis				0.009
No	30	23	7	
Yes	16	6	10	
Distance metastasis				0.030
No	33	24	9	
Yes	13	5	8	

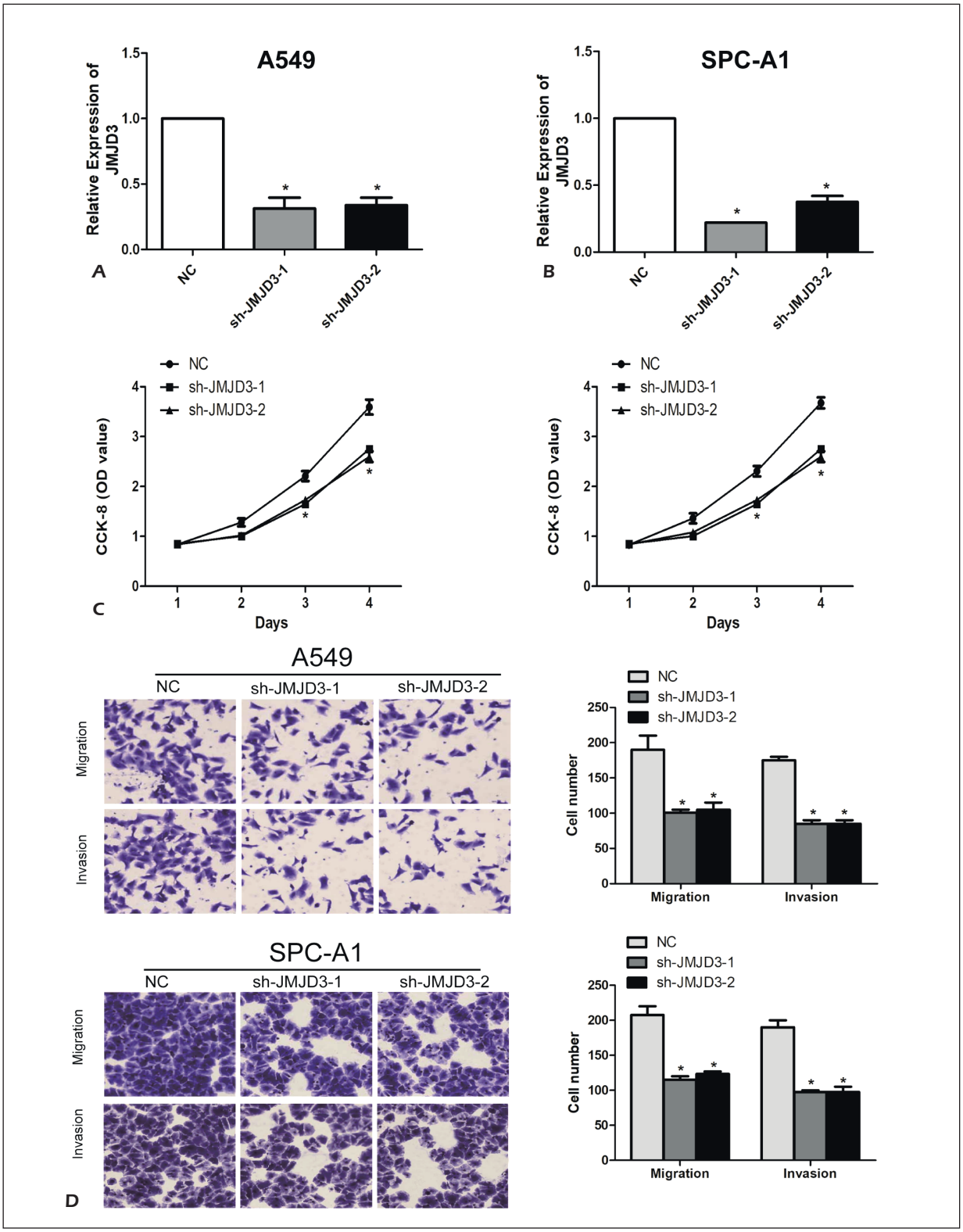

Figure 2. Proliferation, invasion, and migration of non-small cell lung cancer cells were inhibited after silencing JMJD3. **A-B**, qRT-PCR verified the interference efficiency after small interference transfection of JMJD3 in A549 and SPC-A1 cell lines. **C**, CCK-8 assay detected the role of silencing JMJD3 in the cell proliferation of A549 and SPC-A1 cell lines. **D**, Transwell migration and invasion assay detected the invasion and migration of non-small cell lung cancer cells after interference with JMJD3 in A549 and SPC-A1 cell lines (magnification: 20X). Data are mean ± SD, *$p<0.05$.

In addition, the transwell assay detected that the number of cells transferring the membrane of the transwell chamber was conspicuously decreased after knocking down JMJD3, suggesting that the cell migration ability was inhibited. Similarly, the results of the invasion experiment were consistent with the above results.

Knockdown of JMJD3 Changed the Activity of EMT Signaling Pathway

To analyze how JMJD3 promotes cell migration activity and invasiveness, we examined the changes in the expression of key proteins in the EMT pathway such as E-cadherin, N-cadherin, Vimentin, TGF-β, and MMP9 after knockdown of JMJD3. The result of Western blot assay demonstrated that the levels of the above proteins were conspicuously changed after JMJD3 was down-regulated (Figure 3).

JMJD3 Modulated TGF-β Expression in Human Lung Cancer Cells

To further explore the ways in which JMJD3 promotes the malignant progression of NSCLC, we performed the Western blot assay and found that there might exist some possible relationship between JMJD3 and EMT signaling pathway. Furthermore, to further explore the interaction between JMJD3 and TGF-β that belongs to the EGF signaling pathway in NSCLC cells, we added TGF-β cytokines to the NSCLC cell line that had JMJD3 silenced. And the effect of TGF-β cytokines on NSCLC was investigated (Figure 4A, 4B). Subsequently, a transwell migration assay revealed that TGF-β could synergistically promote the migration activity and invasiveness of NSCLC cells with JMJD3 (Figure 4C).

Discussion

Cancer is a serious threat to our quality of life, and it has always been a difficult problem for people to overcome[1-3]. For half a century, lung cancer, the leading malignant tumor, has a poor prognosis and a very low long-term survival[3-5]. Due to various factors such as smoking and passive smoking, smog pollution, poor living habits, and inheritance, the incidence and mortality of lung cancer are showing an increasing trend[5,6]. According to the International Cancer Research Organization, the deaths caused by lung cancer is expected to rise to 10 million per year by 2030. Among the pathological type of lung cancer, about 80% is non-small cell lung cancer (NSCLC), which is easy to invade, migrate, and relapse[7]. Most lung cancer patients are mostly in the advanced stage at the time of diagnosis for they do not have typical clinical symptoms and signs in the early stage, which make it hard to improve treatment effect[10,11]. At present, the specific molecular mechanism of lung cancer occurrence and development, especially lung cancer metastasis, is not very clear.

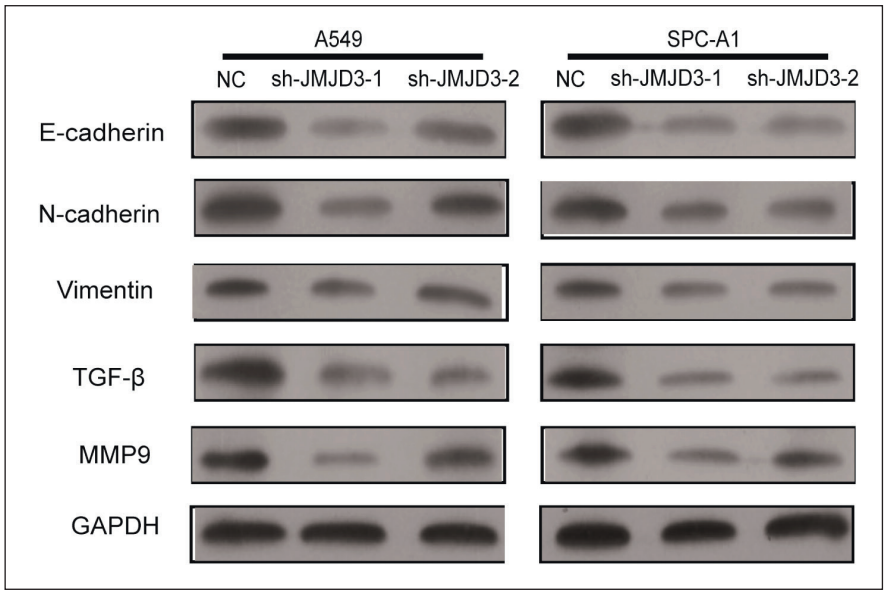

Figure 3. JMJD3 regulated EMT signaling pathway to promote the progression of non-small cell lung cancer. Western blotting verified the expression levels of E-cadherin, N-cadherin, Vimentin, TGF-β, and MMP9 after interference with JMJD3 in A549 and SPC-A1 cell lines.

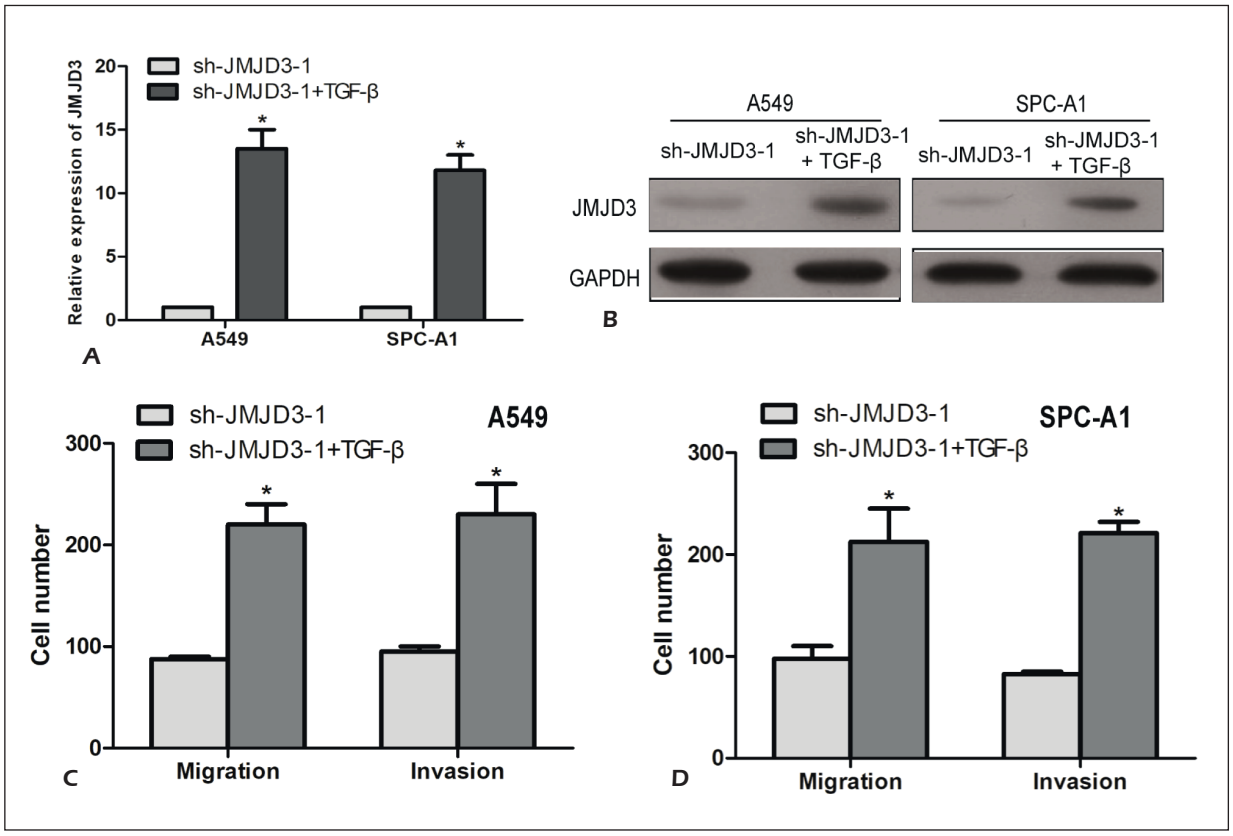

Figure 4. JMJD3 regulated the expression of TGF-β, the key protein in EMT signaling pathway, in non-small cell lung cancer tissues and cell lines. **A**, JMJD3 mRNA expression was detected in cells co-treated with JMJD3 and TGF-β cytokine by qRT-PCR. **B**, JMJD3 protein expression was detected in cells co-treated with JMJD3 and TGF-β cytokine by Western blotting. **C**, Transwell migration invasion assays examined the invasion and migration ability of cells that were co-stimulated by JMJD3 and TGF-β cytokines in AGS and SGC-7901 cell lines (magnification: 20X). Data are mean ± SD, **p* 0.05.

Epithelial-mesenchymal transition (EMT) refers to the process by which epithelial cells acquire the biological properties of certain mesenchymal cells by interacting with the surrounding interstitial cells, which plays a crucial role in the process of body development, tissue damage repair and tissue fibrosis, tumor invasiveness and migratory capacity[23,24]. EMT can change the shape of tumor cells, weaken the adhesion between cells, enhance the motor ability of tumor cells, and promote the spread of tumor cells. Through these methods, the ability of tumor cells to invade and metastasize can be improved, leading to death of cancer patients[24,25]. The level of epithelial cell markers such as E-cadherin is decreased, while that of interstitial cell markers such as vimentin is increased[25].

JMJD3 is a member of the UTM/UTY family of Jmjc domain histone demethylases that specifically catalyze the demethylase of H3K27me3. It can activate gene expression during the extension by removing the inhibitory H3K27me3 marker[20-22]. In our study, we first selected a large number of NSCLC specimens to explore the role of JMJD3 in the development of this cancer. It was found that JMJD3 expression in most tumor tissue samples was increased to varying degrees when compared with adjacent tissues, and a similar result was observed in cell lines. These results indicated that highly-expressed JMJD3 might play an extremely important role in the progression of NSCLC. To verify the effect of JMJD3 on the biological behavior of NSCLC cell lines, we performed CCK8 and transwell assay, and the above experimental results revealed that JMJD3 promoted the migration activity and invasiveness of NSCLC cells. However, the specific molecular mechanism involved in the process still remains elusive.

EMT is a reversible process that plays a vital role in tumor progression and is regulated by post-transcriptional regulation[23-25]. After EMT,

the cells in the form of typical mesenchymal morphology have large intercellular spaces and loose connections. In addition to changes in cell morphology, whether EMT has happened can also be known by analyzing changes in EMT-related marker protein level[24,25]. After the onset of cellular EMT, the levels of markers associated with epithelium are down-regulated, and conversely, those associated with interstitial are up-regulated[25]. Additionally, silencing JMJD3 in cancer cell lines caused an increase in the level of E-cadherin, while the expression of other proteins in the EMT signaling pathway such as N-cadherin, Vimentin, TGF-β, MMP-9 was conspicuously decreased, indicating that JMJD3 could be involved in promoting invasiveness and migratory capacity in NSCLC through EMT signaling pathway. Meanwhile, we have demonstrated that the addition of TGF-β cytokine synergistically promoted the effect of JMJD3 on NSCLC cell function. This evidence suggested that JMJD3 might promote the migration activity and invasiveness of NSCLC cells through EMT signaling pathway.

Conclusions

We found that the expression of JMJD3 in NSCLC tissues and cell lines was conspicuously increased, which might lead to the high incidence of NSCLC lymph node and distant metastasis and poor prognosis of patients with NSCLC. In addition, it was found that JMJD3 might promote the invasion and migration of NSCLC by regulating EMT signaling pathway.

Conflict of Interests

The authors declare that they have no conflict of interest.

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