Jumonji domain containing 2A predicts prognosis and regulates cell growth in lung cancer depending on miR-150

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Abstract. Lung cancer has become the most common cancer worldwide, of which non-small cell lung cancer (NSCLC) accounts for over 80%. Previous studies have shown that the Jumonji domain containing 2A (JMJD2A) was aberrantly expressed in various tumors and involved in the regulation of tumor progression, but the role of JMJD2A on the tumorigenesis in NSCLC and the underlying mechanisms are still unclear. In the present study, we first identified the expression of JMJD2A in NSCLC tissues and cell lines through quantitative RT-PCR (qRT-PCR) and western blotting. Next, the effects of JMJD2A on the progression of NSCLC were analyzed. MTT assay was performed to measure the cell numbers and fluorescence-activated cell sorting (FACS) was adopted to evaluate cell apoptosis. Finally, the relationship between JMJD2A and miR-150 involved in NSCLC was studied. Our results suggested that JMJD2A was significantly overexpressed in NSCLC samples and cell lines. Kaplan-Meier analysis showed that high level of JMJD2A predicted a poor prognosis. Knockdown of JMJD2A inhibited tumor growth and promoted cell apoptosis in NSCLC cells. Additionally, miR-150 was upregulated in NSCLC tissues and positively related with JMJD2A expression. Significant downregulation of miR-150 was observed with JMJD2A knockdown. Furthermore, JMJD2A knockdown inhibited NSCLC cell proliferation while the silencing of miR-150 attenuated the inhibition effect on cell proliferation, suggesting that the effect of JMJD2A on NSCLC cell growth was dependent on miR-150. Thus, our findings identified that JMJD2A played an oncogenic role in NSCLC via regulating miR-150. JMJD2A could possibly serve as a prognostic factor and potential target for NSCLC therapy.

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Introduction

In the past decades, lung cancer has become the leading cause of cancer-related deaths globally, of which non-small cell lung cancer (NSCLC) accounts for over 80% (1-3). Most NSCLC patients are diagnosed at an advanced stage, with a 5-year overall survival rate of 15% because of recurrence and metastasis (4-6). Therefore, exploring the molecular mechanisms underlying the development and progression of NSCLC is urgently required for developing new preventable and therapeutic strategies in clinic.

The accumulation of genetic and epigenetic alterations play important roles in the progression of cancers (7,8). Jumonji domain containing 2A (JMJD2A), one of the histone demethylases, targets histone H3 on lysines 9 and lysines 36 as well as histone H1.4 on lysine 26. Several studies have shown that JMJD2A was aberrantly expressed in lung, gastric, bladder, breast, and other tumors and involved in the regulation of tumor progression (9-16). For instance, JMJD2A is significantly upregulated in gastric cancer, indicating a poor prognosis (11). Additionally, deregulation of JMJD2A is involved in the human carcinogenesis through regulation of the G1/S transition (13). Our previous study uncovered that SIRT2 suppressed NSCLC growth in a JMJD2A-dependent manner and JMJD2A was negatively correlated with SIRT2 in NSCLC (15). However, the role of JMJD2A in NSCLC and the underlying mechanisms are still poorly understood.

MicroRNAs (miRNAs), identified as the important post-transcriptional regulatory factors, participate in several important biological processes, such as proliferation, differentiation, apoptosis and development (17-19). Previous research has reported that miR-150 dysregulation was involved in important processes of cancer, including lung, gastric and prostate cancer (20-23). miR-150 has been confirmed to promote the proliferation and migration of lung cancer cells by targeting SRC kinase signaling inhibitor 1 or p53 (22,23), but its interaction with JMJD2A remains unclear.

We investigated the role of JMJD2A in NSCLC and sought to explore the underlying mechanisms. We identified

significant overexpression of JMJD2A in NSCLC, which was associated with the proliferation and apoptosis of NSCLC cells. Moreover, JMJD2A was also found to function in the progression of NSCLC via regulating miR-150. JMJD2A shows promise as a potential therapeutic target for NSCLC.

Materials and methods

Patients. One hundred and fifty samples of NSCLC tissues and sixteen normal lung tissues were obtained from the Second Affiliated Hospital of Soochow University. The diagnosis of NSCLC was established using World Health Organization morphological criteria. A written form of informed consent was obtained from all patients and donors. The study was approved by the Clinical Research Ethics Committee of the Second Affiliated Hospital of Soochow University (Jiangsu, China).

Cell culture. Two normal lung cell lines (HBE and MRC-5) and four NSCLC cell lines (H460, H1299, A549 and H520) were purchased from the American Type Culture Collection (ATCC). The culture conduction was according to our previous study (15). All cells were maintained at 37˚C with 5% CO_2.

Cell proliferation and apoptosis assay. Cell proliferation was monitored by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation/Viability Assay kit (Sigma, Germany) according to the guidelines.

Apoptosis was evaluated using an Annexin V-Fluos and Propidium Iodide (PI) Apoptosis Detection kit (Sigma) according to the manufacturer's protocol.

Soft sugar colony formation assay. Colony formation assay was based on our previous study (15). A549 cells were suspended in 1.5 ml complete medium supplemented with 0.45% low melting point agarose (Invitrogen, USA). The cells were placed in 35-mm tissue culture plates containing 1.5 ml complete medium and agarose (0.75%) on the bottom layer. The plates were incubated at 37˚C with 5% CO_2 for 2 weeks. Cell colonies were stained with 0.005% crystal violet and analyzed using a microscope.

Tumor xenograft experiments. Xenograft mouse experiments were performed as described previously (15). The tumors were harvested and weighed at the end of the experiments. n=15 in each group.

Luciferase assay. The promoter activity of miR-150 was analyzed using luciferase assay according to a previous study (11). In brief, the human miR-150 promoter was cloned into the pGL4 reporter vector (Promega, USA) to generate a miR-150-luc reporter vector. The cells were co-transfected with miR-150-Luc/p-RL-luc and infected with control and JMJD2A virus. Relative luciferase assays (Promega) were performed as described by the manufacturer.

Quantitative RT-PCR (qRT-PCR) and western blotting. Quantitative RT-PCR (qRT-PCR) and western blotting analysis were performed as described previously (15). The relative fold change of mRNAs was calculated using the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as protein loading control. Primary antibodies against JMJD2A, Bcl-2, Bax, pro-caspase-3, active-caspase-3, GAPDH and the corresponding secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell infection and transfection. For JMJD2A overexpression or knockdown, A549 cell lines were infected with adenovirus expressing ad-JMJD2A or retrovirus expressing sh-JMJD2A, respectively, according to our previous study (15).

For the miR-150 knockdown, miR-150 inhibitor (LNA-anti-miR-150; Exiqon, Denmark) was added to the culture medium according to a previous study (24). The transfection medium was replaced 4 h post-transfection by regular culture medium.

Statistical analysis. All experiments were performed at least three times. Data are shown as mean ± standard deviation (SD). Statistical differences among groups were determined using either Student's t-test or two-way ANOVA. Predictors of differences in overall and disease-free survival were analyzed using Kaplan-Meier analyses. The correlation between JMJD2A and SIRT2 were analyzed using linear regression. Statistical analysis was performed using SPSS software version 16.0. $p<0.05$ was considered statistically significant.

Results

JMJD2A is overexpressed in NSCLC tissues and cell lines. Several studies have reported that JMJD2A was overexpressed in lung cancer (13,14). To confirm whether JMJD2A was aberrantly expressed in NSCLC, we tested the expression of JMJD2A in NSCLC tissues and cell lines through qRT-PCR and western blotting. As shown in Fig. 1A and B, compared with normal lung tissues, JMJD2A was significantly overexpressed in NSCLC tissues. Similarly, significant overexpression of JMJD2A was observed in NSCLC cell lines ($p<0.0001$, Fig. 1C and D).

High level of JMJD2A is correlated with poor prognosis. To explore the relationship between JMJD2A and NSCLC patient prognosis, Kaplan-Meier analysis was performed to assess the rate of overall survival and disease-free survival of patients. We differentiated low-level JMJD2A (n=42) from high-level JMJD2A (n=51) by choosing the mean JMJD2A level of the NSCLC patients as the cut-off point. The results showed that the patients with high level of JMJD2A had poorer overall survival and disease-free survival than those with low level of JMJD2A (Fig. 2), indicating that JMJD2A could serve as a prognostic predictor for NSCLC.

JMJD2A facilitates NSCLC growth and transformation, inhibiting cell apoptosis. The significant overexpression of JMJD2A in NSCLC tissues suggested possible biological significance in tumorigenesis. To investigate the effects of JMJD2A on the tumor progression in NSCLC, we knocked down or overexpressed JMJD2A in A549 cells (Fig. 3D and H). We found that JMJD2A knockdown significantly inhibited

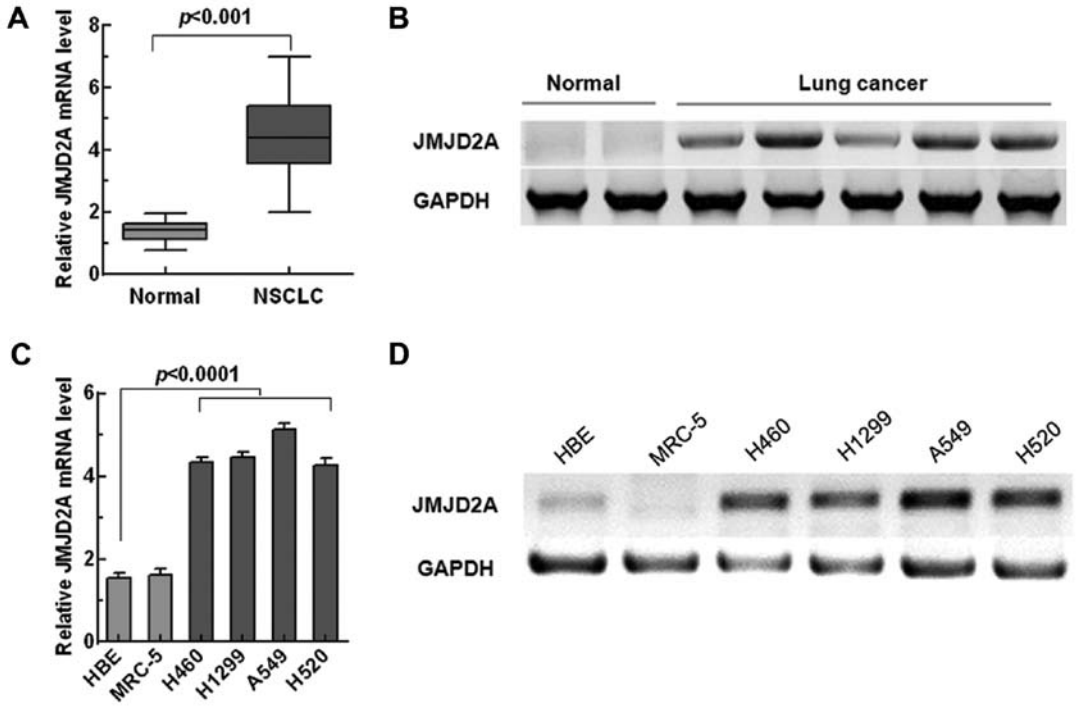

Figure 1. JMJD2A is upregulated in human NSCLC tissues and cell lines. (A) The mRNA level of JMJD2A in NSCLC tissues. (B) The protein level of JMJD2A in NSCLC tissues. n=16 in normal group, n=150 in cancer group. (C) The mRNA level of JMJD2A in lung cancer cell lines. (D) The protein level of JMJD2A in lung cancer cell lines.

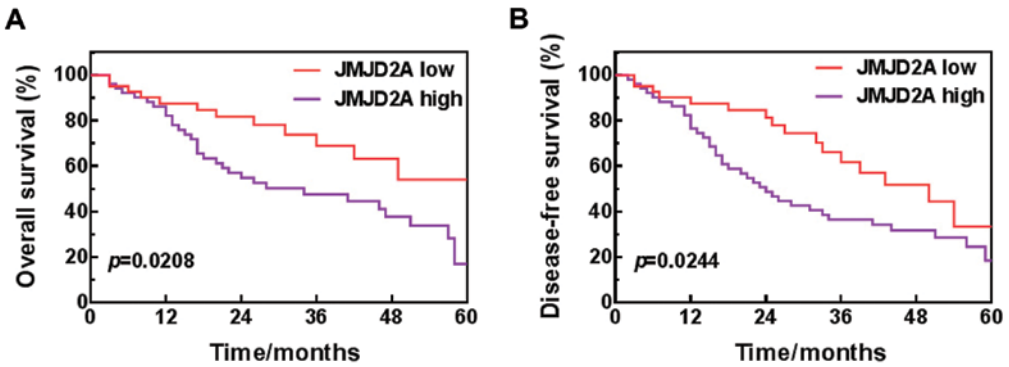

Figure 2. High level of JMJD2A is correlated with poor prognosis. (A) Overall survival. (B) Disease-free survival. The JMJD2A expression was determined using qRT-PCR and the mean JMJD2A level was evaluated. The samples with JMJD2A level lower than the mean (<25%) were included in the low group (n=42), while the others were in the high group (n=51).

cell proliferation and colony formation (Fig. 3A and C) while JMJD2A overexpression promoted the NSCLC growth and transformation (Fig. 3E and G). To confirm the above findings *in vivo*, a xenograft mouse model was adopted. As shown in Fig. 3B and F, JMJD2A knockdown significantly decreased tumor weight while AD-JMJD2A had the opposite effect.

Furthermore, quantitative analysis of apoptotic cells was performed. The results suggested that JMJD2A knockdown significantly accelerated NSCLC cells apoptosis (p<0.001, Fig. 4A). Moreover, JMJD2A knockdown suppressed the expression of the anti-apoptotic proteins Bcl-2 and promoted the levels of the pro-apoptotic proteins (Bax and active-caspase-3) in A549 cells (Fig. 4B). All these results implied that JMJD2A may facilitate tumor growth and regulate cell apoptosis in NSCLC.

JMJD2A positively regulates the expression of miR-150. We have revealed that JMJD2A was correlated with poor prognosis and regulated NSCLC growth, the potential mechanisms underlying the role of JMJD2A in NSCLC were investigated. miR-150 has been reported to promote the proliferation and migration of lung cancer cells (23). Therefore, we investigated whether JMJD2A regulated miR-150 in NSCLC. We first explored the expression of miR-150 in NSCLC tissues. The results showed that miR-150 was markedly upregulated in NSCLC tissues compared with the normal tissues (p<0.001, Fig. 5A). Moreover, linear regression analysis showed that miR-150 level was significantly positively related with JMJD2A level (p<0.0001, Fig. 5B), indicating JMJD2A may regulate miR-150 level in NSCLC. Next, the relationship between JMJD2A and miR-150 was explored. Knockdown of

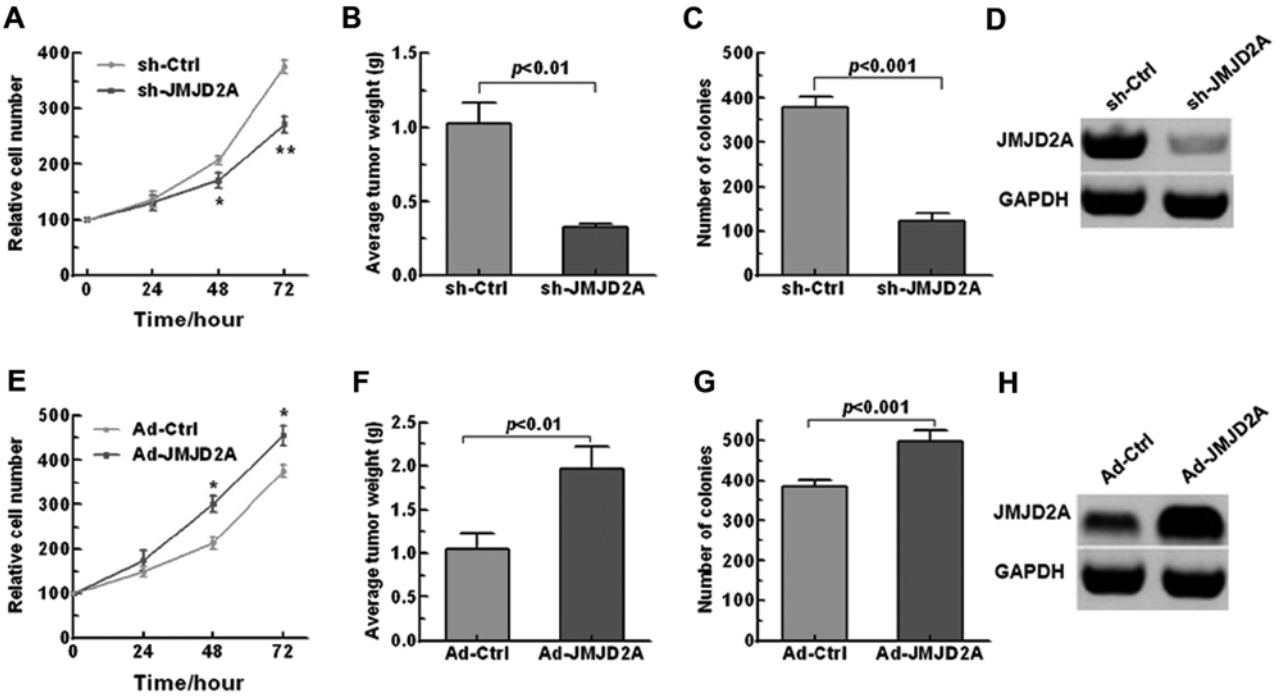

Figure 3. JMJD2A facilitates NSCLC growth and transformation *in vivo* and *in vitro*. (A) JMJD2A knockdown inhibited A549 cell proliferation. *p<0.05, **p<0.01 vs. sh-Ctrl. (B) JMJD2A knockdown suppresses tumor growth *in vivo*; n=15 in each group. (C) JMJD2A knockdown decreased colony formation. (D) The protein level of JMJD2A in A549 after JMJD2A knockdown. (E) JMJD2A overexpression promoted A549 cell proliferation. *p<0.05 vs. Ad-Ctrl. (F) JMJD2A overexpression accelerated tumor growth *in vivo*; n=15 in each group. (G) JMJD2A overexpression increased colony formation. (H) The protein level of JMJD2A in A549 after JMJD2A overexpression.

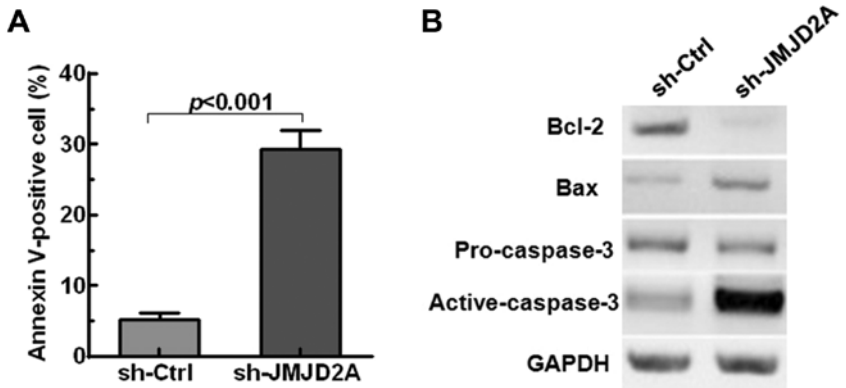

Figure 4. JMJD2A inhibits cell apoptosis in lung cancer. (A) JMJD2A knockdown accelerated apoptosis of A549 cells. (B) JMJD2A knockdown mediated the expression of apoptotic related protein.

JMJD2A in human A549 lung cell lines significantly reduced the miR-150 expression (p<0.001, Fig. 5C). In addition, luciferase analysis also showed that JMJD2A enhanced the promoter activity of miR-150 (Fig. 5D). Our findings revealed that JMJD2A positively regulated the expression of miR-150 in NSCLC.

JMJD2A regulates NSCLC cell growth and apoptosis in a miR-150-dependent manner. We further investigated the regulatory mechanism between JMJD2A and miR-150 in the regulation of NSCLC tumorigenesis. Firstly, LNA-anti-miR-150 was employed to silence miR-150 (p<0.01, Fig. 6A). Furthermore, Fig. 6B shows that miR-150 reduction significantly promoted A549 cell apoptosis. Moreover, we demonstrated that JMJD2A knockdown inhibited NSCLC cell proliferation while silencing the miR-150 attenuated the inhibition effect on cell proliferation (Fig. 6C), suggesting that the effect of JMJD2A on NSCLC cells growth was dependent on miR-150. In addition, Kaplan-Meier survival analysis showed that high miR-150 level predicted a poor overall survival (Fig. 6D). Taken together, these results revealed that JMJD2A regulated the tumor progression in NSCLC in a miR-150-dependent manner.

Discussion

Notwithstanding that JMJD2A was aberrantly expressed in various tumors and involved in the regulation of tumor progres-

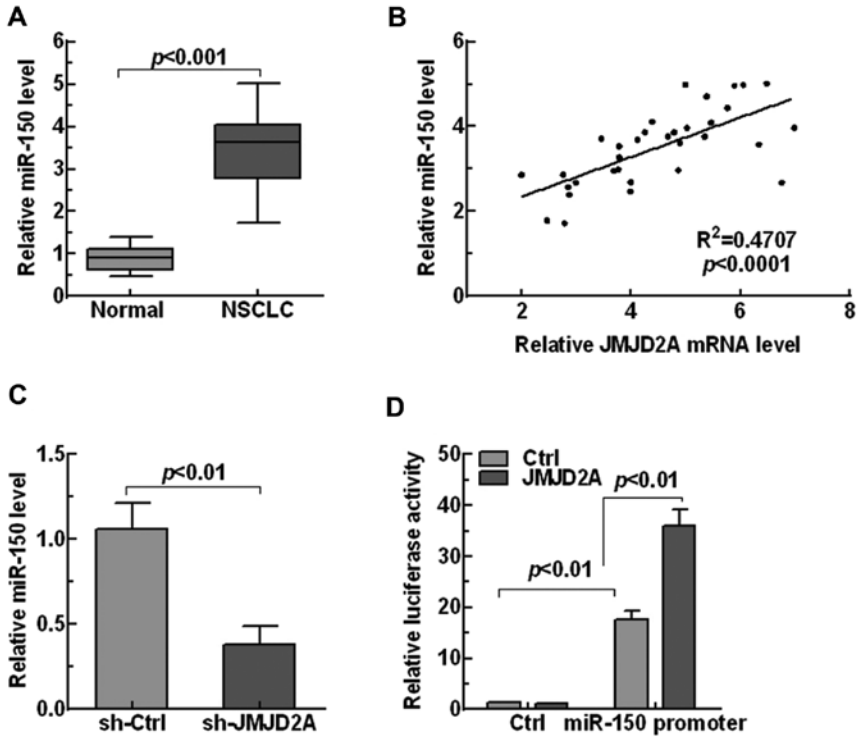

Figure 5. JMJD2A regulates miR-150 in NSCLC. (A) miR-150 was upregulated in human NSCLC tissues; n=16 in normal group, n=33 in NSCLC group. (B) miR-150 expression was correlated with the JMJD2A level; n=33. (C) JMJD2A knockdown repressed the expression of miR-150 in A549 cells. (D) JMJD2A increased the promoter activity of miR-150 in A549 cells.

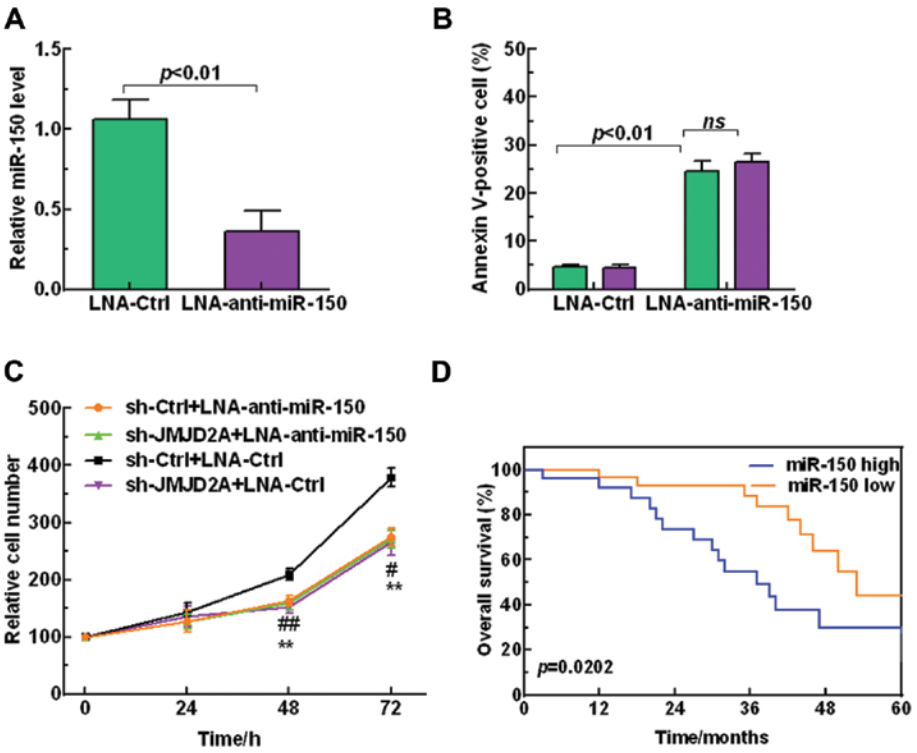

Figure 6. miR-150 knockdown is essential to block the effects of JMJD2A on NSCLCs. (A) miR-150 was knocked down in NSCLC. (B) The miR-150 knockdown blocked the effect of JMJD2A on the lung cancer cell apoptosis. ns, no significance. (C) The miR-150 knockdown eliminated the effect of JMJD2A on lung cancer cell proliferation. **p<0.01, sh-JMJD2A+LNA-Ctrl vs. sh-Ctrl+LNA-Ctrl; $^{\#}$p<0.05, $^{\#\#}$p<0.01, sh-Ctrl+LNA-Ctrl vs. sh-Ctrl+LNA-antimiR-150. (D) High level of miR-150 predicted poor survival. n=25 in miR-150 high group, n=27 in miR-150 low group.

sion (9-16), its role in NSCLC growth was still unknown. Our previous study uncovered that SIRT2 suppressed NSCLC growth in a JMJD2A-dependent manner, which was negatively correlated with JMJD2A in NSCLC (15). Thus, we

conjectured that JMJD2A may participate in the regulation of the NSCLC growth. To determine this hypothesis, we first analyzed the expression of JMJD2A in NSCLC tissues and cell lines. Consistent with the findings of previous research (13-15), JMJD2A was overexpressed in NSCLC tissues and cell lines. We also found that high level of JMJD2A was associated with a poor prognosis in NSCLC. Additionally, JMJD2A was knocked down or overexpressed to explore its functional role in NSCLC progression. Our results showed that JMJD2A overexpression promoted cell proliferation, colony formation while inhibited cell apoptosis in NSCLC. Taken together, JMJD2A showed porential to play a pivotal role in the tumorigenesis of NSCLC.

Furthermore, we explored the potential mechanisms underlying the role of JMJD2A in NSCLC. Several miRNAs have been reported to function as oncogenes or tumor suppressors in various tumors (25-29). Recent studies have revealed that miR-150 was aberrantly expressed in various types of diseases, including pediatric intestinal Burkitt's lymphoma, irritable bowel syndrome, dengue haemorrhagic fever, acute myeloid leukemia, systemic sclerosis, and gastric cancer (20,30-34). Importantly, miR-150 was significantly upregulated in lung cancer tissues and promoted the proliferation and migration of lung cancer cells (22,23). Thus, we speculated that JMJD2A may have correlations with miR-150 in NSCLC. Our results showed that miR-150 was markedly upregulated in NSCLC tissues and positively related with JMJD2A. In addition, miR-150 was significantly downregulated in NSCLC cells with JMJD2A knockdown, implying that miR-150 may be regulated by JMJD2A in NSCLC. Luciferase analysis also suggested that JMJD2A enhanced the promoter activity of miR-150. All these findings indicated that miR-150 was a target of JMJD2A in NSCLC. Besides, silencing the miR-150 significantly promoted cell apoptosis in A549 cells, which agreed with the results of previous studies (22,23). To further confirm whether JMJD2A regulated NSCLC growth through miR-150, we knocked down JMJD2A and miR-150, respectively, or simultaneously in NSCLC cells. Obviously, JMJD2A knockdown inhibited NSCLC cell proliferation while silencing miR-150 attenuated the inhibition effect on cell proliferation, indicating that miR-150 was critically essential for the function of JMJD2A in NSCLC. The results suggested that JMJD2A regulated NSCLC growth by regulating miR-150.

Our previous study reported that SIRT2 suppressed NSCLC growth in a JMJD2A-dependent manner (15). In the present study, we also identified that JMJD2A promoted NSCLC growth by regulating miR-150. Unfortunately, the deficiency is, the relationship between SIRT2 and miR-150 remaining largely unknown, which needs our further attention.

In summary, this study demonstrates that JMJD2A contributes to tumorigenesis in NSCLC by regulating miR-150. Additionally, JMJD2A overexpression is associated with a poor prognosis for NSCLC patients. JMJD2A may serve as a potential therapeutic target for NSCLC.

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