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Research article

METTL3-mediated m^{6}A methylation of DNMT1 promotes the progression of non-small cell lung cancer by regulating the DNA methylation of FOXO3a

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ABSTRACT

Background: The aim of this study was to investigate the effect of DNA methylation of Fork Head Box O3 (FOXO3a) on the process of epithelial-mesenchymal transition (EMT) in non-small cell lung cancer (NSCLC).

Methods: The expressions of FOXO3a, DNA methyltransferase 1 (DNMT1), METTL3, and EMT-related proteins (E-cadherin and N-cadherin) were measured. The influence of 5-Aza-dC and DNMT1 on the methylation level in the promoter region of FOXO3a was examined through the application of methylation-specific PCR (MSP). Chromatin immunoprecipitation (ChIP) was employed to detect binding between DNMT1 and the FOXO3a promoter. Methylated RNA immunoprecipitation (MeRIP) was utilized to evaluate the level of DNMT1 N6-methyladenosine (m6A) methylation. The assessment of cell viability and invasion abilities of A549 cells was performed using Cell Counting Kit-8 (CCK-8) and Transwell assays, respectively. NSCLC xenograft mouse models were established by subcutaneously injected treated A549 cells into nude mice.

Results: The expression levels of DNMT1 and DNA methylation level FOXO3a were found to be significantly increased, whereas FOXO3a expression was considerably decreased in NSCLC cell lines and NSCLC tumor tissues. Both 5-Aza-dC treatment and DNMT1 knockdown resulted in the down-regulation of DNA methylation levels of FOXO3a while simultaneously up-regulating the expression of FOXO3a. A ChIP assay demonstrated that DNMT1 has the ability to bind to the promoter region of FOXO3a. Furthermore, the knockdown of DNMT1 promoted E-cadherin expression, but inhibited expression of N-cadherin, cell viability, and invasion ability. However, the knockdown of FOXO3a hindered the effect of DNMT1 knockdown on EMT, cell viability, and invasion ability of A549 cells. This was evidenced by decreased E-cadherin expression and increased N-cadherin expression, as well as increased cell viability and invasion ability. Increased expression of DNMT1 resulted from m6A methylation of DNMT1, which was mediated by METTL3. Overexpression of DNMT1 decreased of E-cadherin expression while increased N-cadherin expression, cell viability, and invasion ability in METTL3-shRNA treated A549 cells. In xenograft mouse models, DNMT1 knockdown significantly reduced tumor volumes and tumor weight. DNMT1 knockdown upregulated the expression of FOXO3a and E-cadherin, while downregulated N-cadherin expression *in vivo*.

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Conclusion: METTL3-mediated m6A methylation of DNMT1 up-regulates FOXO3a promoter methylation, thereby promoting the progression of NSCLC.

1. Introduction

The most lethal form of cancer worldwide remains lung cancer, which has been divided into two histological subgroups: non-small cell lung cancer (NSCLC) and small-cell lung cancer. With a 5-year survival rate of less than 15%, NSCLC accounts for 80%–85% of all lung cancers [1]. Therefore, the development of new molecular techniques for diagnosing, treating, and predicting the prognosis of NSCLC patients remains a crucial clinical requirement [2].

Previous research indicates that epigenetic changes contribute to the onset and progression of malignancies. Epigenetic modification mainly influences gene function and expression through DNA methylation, histone alterations, and chromatin remodeling, thereby impacting tumor progression [3]. N6-methyladenosine (m^6A) is a frequent internal alteration of mRNA that affects RNA processing, localization, translation, and eventual degradation, consequently influencing gene expression. This epigenetic mark serves as "writers," "erasers," and "readers" for each of these processes [4,5]. To participate in the biological processes and chemotherapy resistance of NSCLC cells, m^6A methylation-related factors can be utilized as either cancer-promoting factors or tumor suppressor factors. It is believed that studying the function and mechanism of m^6A RNA methylation in NSCLC could lead to novel diagnostic and therapeutic approaches. NSCLC is a highly fibrotic cancer with a significant amount of connective tissue-promoting mesenchyme. One of the primary methods of tumor invasion is epithelial-mesenchymal transition (EMT), which is directly associated with low survival rates in patients [6].

DNA methyltransferase (DNMT1) is an important enzyme that regulates gene methylation and cytosine methylation transfer activity. By controlling the intracellular methylation process, DNMT1 may contribute to the incidence and progression of tumors [7]. In breast cancer and NSCLC, DNMT1 is up-regulated, which increases the methylation of tumor suppressor genes and suppresses their expression, thereby accelerating the growth of tumors [8]. On chromosome 6q21, the presence of Fork Head Box O3 (FOXO3), a member of the mammalian Fox O subfamily, has been identified [9]. It plays a crucial role in transcription, which regulates a number of physiological and pathological processes by activating target genes involved in cell proliferation, survival [10], and DNA damage repair [11,12]. It also promotes cell apoptosis, proliferation [13], and cell cycle progression [14]. Down-regulation of FOXO3a has been linked to tumor growth, progression, and poor prognosis in several human cancers [15,16]. The FOXO3a promoter region can undergo significant methylation by DNMT1, leading to its suppression. This suppression promotes the characteristics of breast cancer stem cells and tumor formation while inhibiting the expression of FOXO3a [11]. It is currently unknown whether DNMT1 can contribute to invasion and metastasis in NSCLC by increasing methylation of the FOXO3a promoter and inhibiting its expression. The m^6A modification of DNMT1 has not been reported. But neither the m^6A modification of DNMT1 nor the control of DNMT1's m^6A methylation by METTL3 in NSCLC have been documented.

The "writers" complex, which includes METTL3, METTL14, and WT1 associated protein (WTAP) as its primary components, affects translation, mRNA degradation, and RNA stability to regulate gene expression levels. Consequently, human malignancies will manifest and progress when METTL3 is dysfunctional [17]. According to a previous study, METTL3 enhances Bcl-2 expression through m^6A alteration, thereby increasing the survival and migratory capacity of NSCLC cells [18]. When the expression of FOXO3a was down-regulated due to increased methylation of the FOXO3a promoter by DNMT1, it encouraged invasion and EMT in NSCLC.

2. Materials and methods

2.1. Clinical sample collection

Twelve tumor tissues and pair-matched adjacent non-neoplastic tissue were collected from patients with NSCLC after surgical resection at Xi 'an International Medical Center Hospital. This study was approved by the Ethics Committee of Xi 'an International Medical Center Hospital. Informed consent was obtained from all patients.

2.2. Cell lines and culture

Normal human lung epithelial cells BEAS-2B (iCell-h023) and NSCLC cell lines A549 (iCell-h011), NCI–H1299 (iCell-h153), NCI–H1650 (iCell-h155-001b), and NCI–H1975 (iCell-h156) were obtained from iCell (Shanghai, China). They were cultivated in Dulbecco's modification of Eagle's medium Dulbecco (DMEM) (iCell-0001), F–12K (iCell-0007), or RPMI-1640 (iCell-0002) media supplemented with 10% phosphate buffer saline (PBS) and 100 U/mL penicillin-streptomycin.

2.3. Methylation-specific PCR (MSP)

MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) was used to identify the CpG islands in the FOXO3a promoter region. A549 cells' genomic DNA has been removed using a DNA extraction kit. Purified modified DNA was provided for methylation-specific polymerase chain reaction (MSP) or bisulfite sequencing with methylated and non-methylation-specific markers,

along with 100 ng of bisulfite-modified DNA. After designing and selecting the primer, MSP is performed using standard PCR conditions. The Gene Amp DNA Amplification Kit (a2361, Promega, Beijing, China) and Amp LiTaq Gold polymerase (EM101, Tiangen, Beijing, China) were the components of the PCR kit. The PCR amplification settings were as follows: 95 °C for 5 min, 60 °C for 30 s, and 72 °C for 20 s for 35 cycles, with a final extension at 72 °C for 5 min. After the reaction, 10 μL of reaction products were analyzed using agarose gel electrophoresis.

2.4. Infection and transfection

Upon reaching approximately 75% confluence, A549 cells (at a density of 1×10^5/well) were digested, counted, and then planted in 24-well plates. The cells were cultured overnight at 37 °C. One mL of reduced serum medium was added to a 1.5 mL centrifugation tube, followed by the addition of the infectious agent and virus at 37 °C. Eight hours later, in an incubator with 5% CO_2, the culture medium was completely changed. Cells were collected 72 h after infection, and the expression of mRNA and protein was identified using reverse transcription polymerase chain reaction (RT-qPCR) and Western blot to determine the effectiveness of the infection. Puromycin was then used for screening. Stable strains were used to produce cells that were either inhibited or overexpressed. All of the lentiviruses used in this experiment (LV-sh-NC, LV-sh-DNMT1, LV-sh-FOXO3a, LV-sh-METTL3, LV-OE-NC, and LV-OE-DNMT1) were created by OBIO Technology (Shanghai, China).

2.5. Cell viability

To detect cell viability, the Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China) was used. In a 96-well plate, approximately 5×10^3 A549 cells were inserted in the center of each well. At 24 h, each well received 10 μL of CCK-8 solution, which was then incubated for 1 h. A microplate (Thermo Fisher Scientific, Waltham, MA, USA) was utilized to measure the absorbance at 450 nm for each well. Three replicates of each experiment were carried out.

2.6. Transwell assay

Two hundred μL of the treated A549 cells were added to the upper Transwell chamber, while 600 μL of media containing 20% fetal bovine serum was added to the lower chamber. The treated A549 cells were diluted to a concentration of 1×10^5/mL in serum-free F–12K medium before being added. Using sterile forceps, the top chamber was slowly submerged in the bottom chamber. The 24-well plate containing a chamber was incubated for 24 h at 37 °C. The liquid was taken from the upper chamber after 24 h and poured three times into a hole containing 600 μL of PBS. The upper chamber was examined and photographed using an electron microscope after being stained with crystal violet. A total of three experiments were conducted.

2.7. Reverse transcription polymerase chain reaction (RT-qPCR)

According to the manufacturer's instructions, total RNA was collected from the cultivated cell lines using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Ultraviolet spectroscopy was employed to calculate the concentration of RNA using the NanoDrop 2000. The Roche Reverse Transcription Kit was then used to reverse transcribe the RNA into cDNA. The endogenous control used for studying gene expression was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The fold change of gene expression was calculated using the $2^{-\triangle\triangle Ct}$ method. The primer sequences were as follows: METTL3 Forward: 5′- GTCCATCTGTCTTGCCATCTC -3′, Reverse: 5′- GAGACCTCGCTTTACCTCAATC -3'; FOXO3a Forward: 5′- AGCCTAACCAGGGAAGTTTG -3′, Reverse: 5′- GACTCACT-CAAGCCCATGTT -3′, DNMT1 forward: 5′ CCAAAGAACCAACACCCAAAC-3′, Reverse: 5′ CTCATCTTTCTCGTCTCCATCTTC-3'.

2.8. Western blot analysis

A protease and phosphatase inhibitor cocktail was added to the Radio Immunoprecipitation Assay (RIPA) buffer to lyse the cells. The proteins were electron transferred to an immunoblot polyvinylidene fluoride (PVDF) membrane following SDS-PAGE. Five percent body surface area (BSA) was then used to block membranes in protein quantitative analysis using ImageJ. Primary rabbit antibodies against METTL3 (1: 1000 dilution; ab195352, Abcam, Cambridge, UK), FOXO3a (1: 1000 dilution; ab109629, Abcam), DNMT1 (1: 1000 dilution; ab188453, Abcam), E-cadherin (1: 1000 dilution; ab40772, Abcam), N-cadherin (1: 5000 dilution; ab76011, Abcam), and GAPDH (1: 500 dilution; ab8245, Abcam) were used. Horseradish peroxidase (HRP)-labeled secondary antibody goat anti-rabbit IgG (1: 2000 dilution; ab205718, Abcam) was also used. The ratio of the gray value of the target band to that of the internal reference GAPDH band served as a measure of protein expression.

2.9. Chromatin immunoprecipitation (ChIP) qPCR assay

The Chromatin Immunoprecipitation (ChIP) Assay Kit (P2078, Beyotime, China) was used to carry out to identify the binding of DNMT1 and the promoter region of FOXO3a (selected area: 951 to −1050 at the promoter region). Cells were briefly rinsed in a phosphate-buffered solution containing 1 mM phenylmethanesulfonyl fluoride (PMSF) fluoride, followed by centrifugation at 800*g* for 2 min to fully precipitate the cells. The supernatant was then removed, treated with lysis buffer, and sonicated. The supernatants from the sonicated samples were collected by centrifuging them at 12,000 g for 5 min, and 1.8 mL of ChIP dilution buffer, containing 1 mM

PMSF, was added to dilute the sample to a final volume of 2 mL. A 20-μL sample was taken as input for follow-up testing. The protein A + G and primary antibody DNMT1 (1:50) were then added to the supernatant. The samples were then centrifuged for 1 min at 1000 g. After adding 250 μL of elution buffer to the sediment twice for elution centrifugation to obtain 500 μL of supernatant, the cross-linking between the supernatant and the protein and genomic DNA in the input sample was eliminated, and PCR assays were performed. The primer sequence for FOXO3a is as follows: Forward: 5′- TCGCGTGTGGTGCATTC -3′, Reverse: 5′- CATCTGCACAAGTCCTTATTCT -3’.

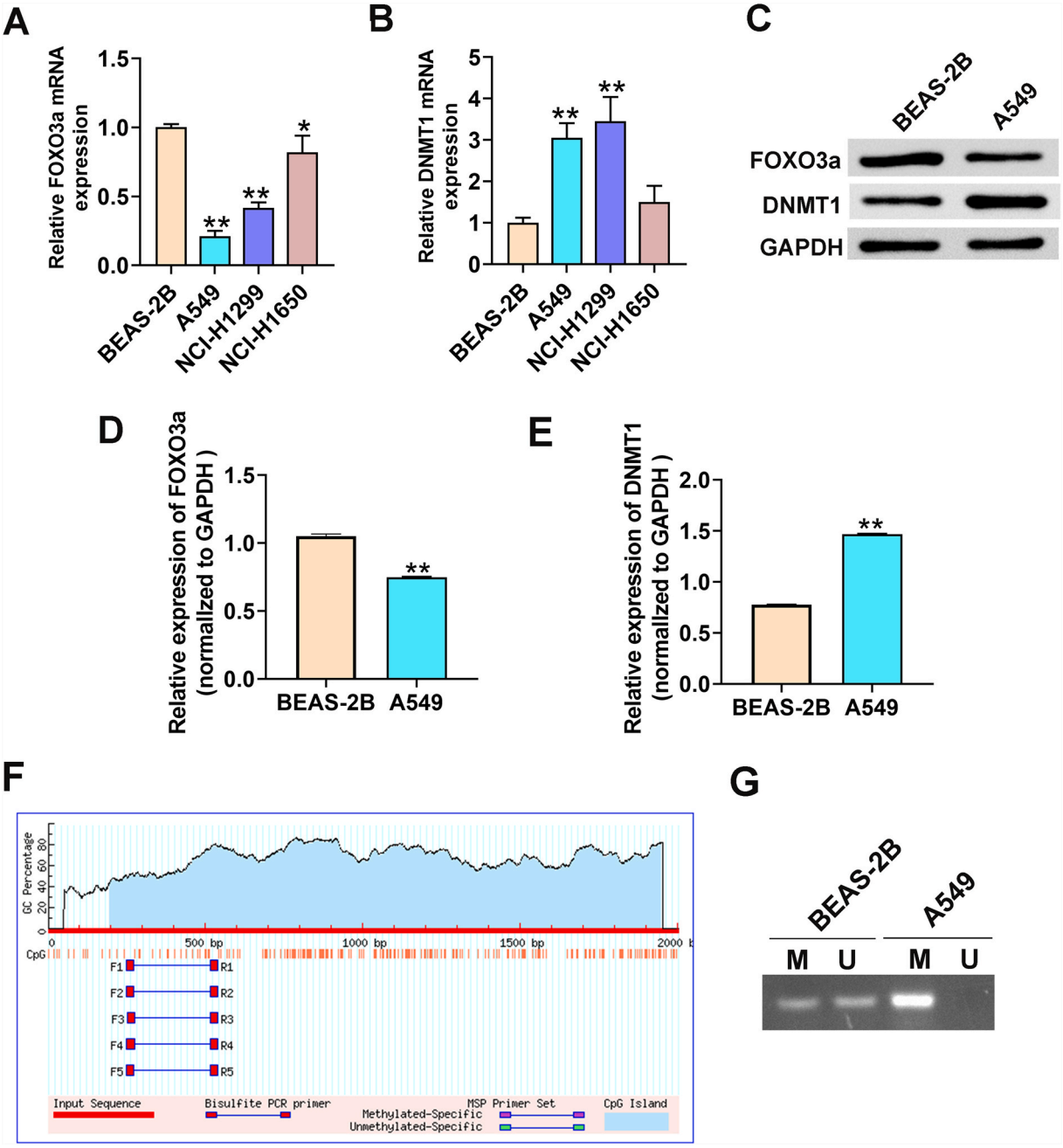

Fig. 1. FOXO3a gene expression was down-regulated in NSCLC cells, while FOXO3a promoter methylation level and DNMT1 expression were up-regulated. (A) FOXO3a mRNA expression was measured by RT-qPCR analysis in BEAS-2B and NSCLC cells. (B) DNMT1 mRNA expression was measured by RT-qPCR analysis in BEAS-2B and NSCLC cells. (C) Protein expression of FOXO3a and DNMT1 in BEAS-2B and A549 cells was analyzed using Western blot analysis. (D) The graph shows the FOXO3a expression (normalized to GAPDH) in the BEAS-2B and A549 cells. (E) The graph shows the DNMT1 expression (normalized to GAPDH) in the BEAS-2B and A549 cells. (F) MethPrimer was used to identify the CpG islands in the FOXO3a promoter region (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). (G) FOXO3a promoter methylation levels in BEAS-2B and A549 cells were detected using MSP. $*P < 0.05$, $**P < 0.01$ vs. BEAS-2B.

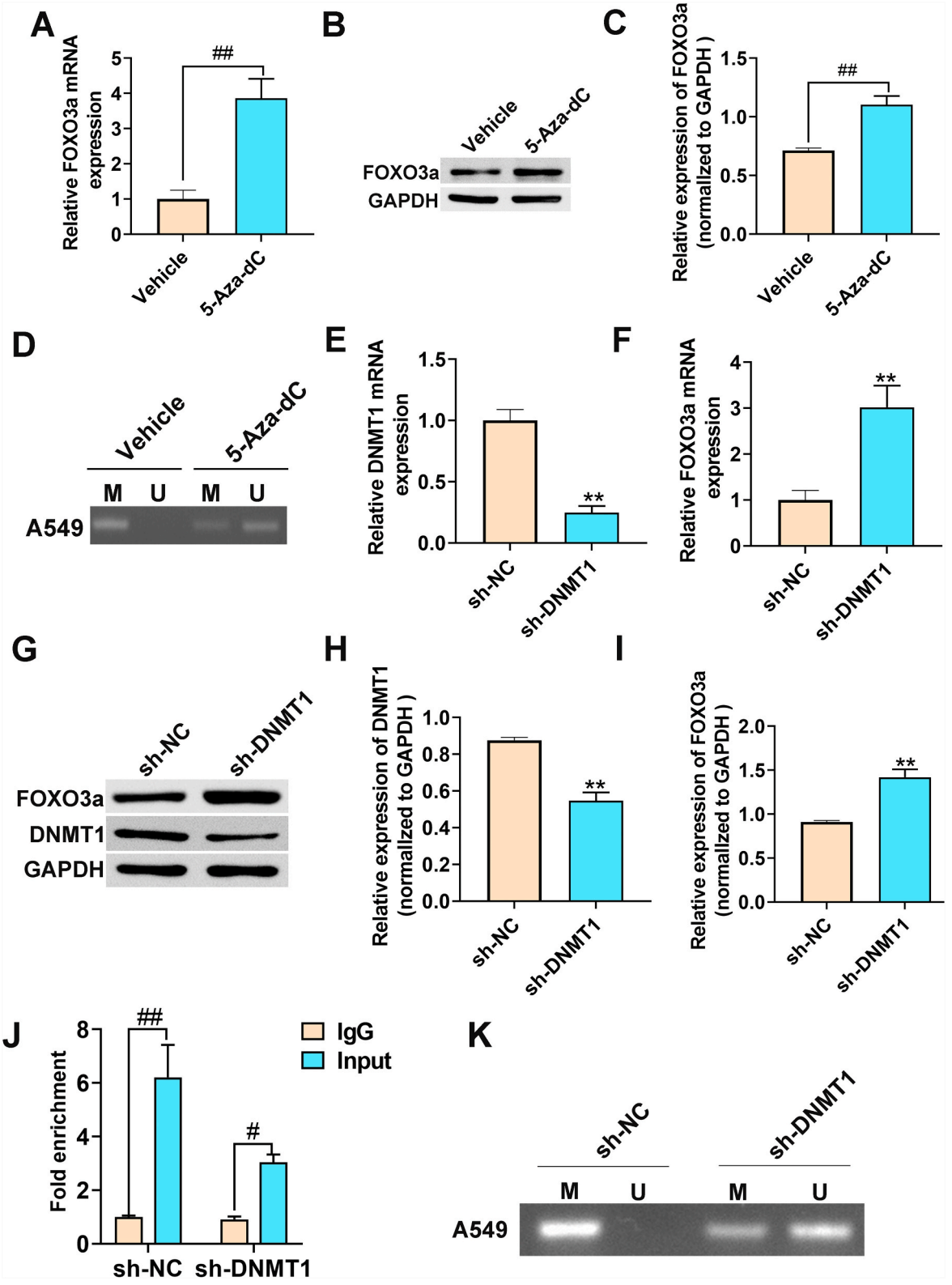

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Fig. 2. DNMT1 knockdown up-regulated FOXO3a expression by inhibiting FOXO3a methylation. A549 cells were treated with 15 μM of 5-Aza-dC for 48 h [19]. (A) The mRNA expression of FOXO3a was detected using RT-qPCR. (B–C) Expression of the FOXO3a protein in A549 cells treated with 5-Aza-dC. (D) In A549 cells treated with the methyltransferase inhibitor 5-Aza-dC, the FOXO3a promoter was found to be methylated using the MSP method. (E–F) RT-qPCR was used to identify the mRNA expression of DNMT1 and FOXO3a in A549 cells infected with sh-NC and sh-DNMT1, respectively. GAPDH functioned as the internal control. (G–I) DNMT1 and FOXO3a protein expression was examined using Western blot analysis in A549 cells that had been infected with sh-NC and sh-DNMT1, respectively. GAPDH plays a role as an internal control. (J) The binding of DNMT1 to the FOXO3a promoter region was determined by ChIP. (K) FOXO3a promoter methylation was assessed in A549 cells infected with sh-NC and sh-DNMT1 using the MSP assay. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; $^{**}P < 0.01$ vs. sh-NC.

2.10. Methylated RNA immunoprecipitation (MeRIP) assay

sh-NC or sh-METTL3 was transfected into A549 cells. The methylated RNA immunoprecipitation (MeRIP) reagent was used with 30 μg of total RNA. Magna ChIP Protein A/G Magnetic Beads were first re-suspended. Then, the RNA was cultured with an m6A antibody (1: 500; ab151230, Abcam) or an IgG antibody (1: 100; ab109489, Abcam) overnight at 4 °C. After that, the mixture was incubated with protein A + G magnetic beads for 1 h. Finally, the complex was incubated with an immunoprecipitation (IP) buffer overnight. RNA was extracted and purified. DNMT1 was examined using qRT-PCR. The primer sequence for DNMT1 (selected region: 4340–4470) is as follows: Forward: 5′- CTGTAAGGACATGAGTGCATTG -3′, Reverse: 5′- CCTCGATGTTGGGCAGAT -3’.

2.11. Animal experiments

BALB/c nude mice (4-week-old) were purchased from the Animal Experiment Center of Xi’an Jiaotong University (Xi’an, China). To establishment the xenograft models, twenty mice were randomly divided into four groups, each of which contained five nude mice. In the sh-NC group, 4×10^6 A549 cells infected with LV-sh-NC were diluted in 100 μl of PBS and injected subcutaneously into nude mice. In the sh-DNMT1 group, 4×10^6 A549 cells infected with LV-sh-DNMT1 were diluted in 100 μl of PBS and injected subcutaneously into nude mice. In the sh-DNMT1 + sh-FOXO3a group, 4×10^6 A549 cells infected with LV-sh-DNMT1 and LV-sh-NC were diluted in 100 μl of PBS and injected subcutaneously into nude mice. In the sh-DNMT1 + sh-FOXO3a group, 4×10^6 A549 cells infected with LV-sh-DNMT1 and LV-sh-FOXO3a were diluted in 100 μl of PBS and injected subcutaneously into nude mice. The volume of the tumors was measured every 3 days for 21 days. 21 days later, the mice were sacrificed, and the tumor xenografts were collected for tumor weight analyses and Western blot analyses. All animal experiment procedures were approved by the Animal Care and Use Committee of Xi ’an International Medical Center Hospital. The animal experiments strictly followed the ethical guidelines.

2.12. Statistical analysis

Data were analyzed using a two-tailed Student’s unpaired *T*-test and a one-way analysis of variance (ANOVA). A significance level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. FOXO3a gene expression was down-regulated in NSCLC cells, while FOXO3a promoter methylation level and DNMT1 expression were up-regulated

Compared to the BEAS-2B cells, the FOXO3a mRNA level was significantly lower in A549, NCI–H1299, and NCI–H1650 cells (Fig. 1A). In contrast, A549, NCI–H1299, and NCI–H1650 cells had significantly higher DNMT1 mRNA levels (Fig. 1B). A549 cells were selected for the tests based on the aforementioned results. Subsequently, the FOXO3a protein level was significantly decreased, while the DNMT1 protein level was significantly increased in A549 cell lines compared to BEAS-2B cells (Fig. 1C–E). As predicted by the MethPrimer website, there was a highly methylated site in the FOXO3a promoter region (Fig. 1F). MSP demonstrated a higher methylation level of FOXO3a in A549 cells compared to BEAS-2B cells (Fig. 1G).

3.2. DNMT1 knockdown up-regulated FOXO3a expression by inhibiting FOXO3a methylation

It was shown that 5-Aza-dC increased FOXO3a mRNA and protein levels (Fig. 2A–C). In addition, MSP assays confirmed that 5-Aza-dC could decrease the methylation level of FOXO3a (Fig. 2D). Subsequently, A549 cells were stably transfected with lentivirus for DNMT1 knockdown. The levels of sh-DNMT1 group DNMT1 mRNA and protein were lower than those of the sh-NC group, while FOXO3a mRNA and protein levels were higher in the sh-DNMT1 group than in the sh-NC group (Fig. 2E–I). However, knockdown of DNMT3A and DNMT3b using respectively small interference RNA has no effect on FOXO3a protein expression (Supplementary Fig. 1). Moreover, the ChIP assay discovered that DNMT1 binds to the FOXO3a promoter in A549 cells (Fig. 2J). Additionally, the MSP assay was used to determine the methylation status of FOXO3a in the sh-NC group and the sh-DNMT1 group. According to the findings, the DNA methylation level of FOXO3a was significantly reduced in the sh-DNMT1 group compared to the sh-NC group (Fig. 2K).

Clinical study demonstrated that the mRNA expression of FOXO3a was significantly decreased in NSCLC tumor tissues when compared to the adjacent normal tissues (Fig. 3A). This result was further confirmed by Western blot assay. As shown in Fig. 3B, FOXO3a expression was decreased whereas DNMT1 expression was increased in NSCLC tumor tissues when compared to the adjacent

normal tissues. Furthermore, the DNA methylation level of FOXO3a was higher in the tumor tissues than in the adjacent normal tissues (Fig. 3C).

3.3. DNMT1 affects NSCLC cell invasion and EMT by regulating FOXO3a expression

To investigate the influence of DNMT1 on the EMT of NSCLC and its underlying mechanism, FOXO3a was knocked down in conjunction with DNMT1. The FOXO3a mRNA and protein expression in A549 cells was significantly decreased in the sh-DNMT1+sh-FOXO3a group compared to the sh-DNMT1+sh-NC group (Fig. 4A–C). Cell viability and invasion rates were both significantly decreased after DNMT1 knockdown. The sh-DNMT1+sh-FOXO3a group's cell viability and cell invasion number were higher than those of the sh-DNMT1+sh-NC group (Fig. 4D–E). Additionally, the expression of the E-cadherin protein was much higher in the sh-DNMT1 group compared to the sh-NC group, while the expression of the N-cadherin protein was significantly decreased. On the other hand, compared to the sh-DNMT1+sh-NC group, the expression of E-cadherin protein was significantly lower, and the N-cadherin protein expression was significantly higher in the sh-DNMT1+sh-FOXO3a group (Fig. 4F–H).

3.4. The N6-Methyladenosine (m^6A) modification level expression of DNMT1 and METTL3 in NSCLC cells was significantly increased

The m^6A methylation level of DNMT1 was predicted using the Sequence-Based RNA Adenosine Methylation Site Predictor (SRAMP) database website. The finding that DNMT1 mRNA had 10 m^6A sites with very high confidence suggested a potential

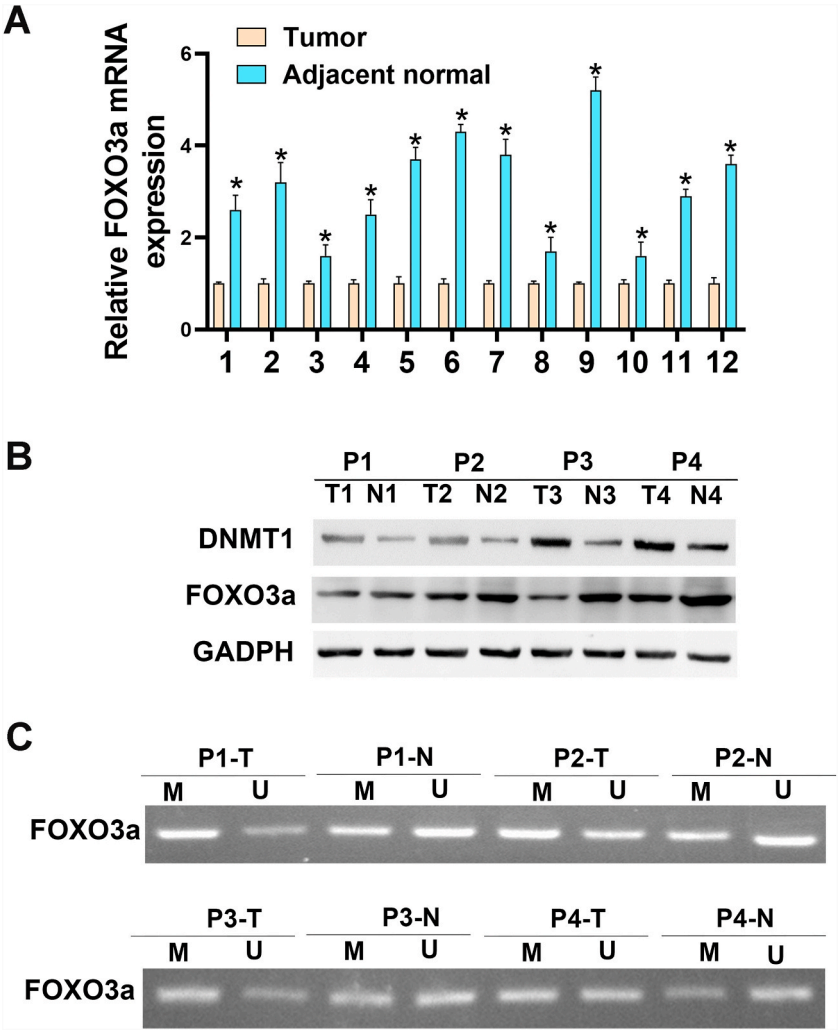

Fig. 3. The DNMT1, FOXO3a and FOXO3a methylation expression in NSCLC tumor tissues. (A) FOXO3a mRNA level in NSCLC tumor and adjacent normal tissue was examined by RT-qPCR. (B) DNMT1 and FOXO3a protein expression in NSCLC tumor and adjacent normal tissue was examined using Western blot analysis. (C) FOXO3a promoter methylation level was measured by MSP assay in tumor and adjacent normal tissue from NSCLC patients. M represents for methylation, U for unmethylation, T for tumor and N for adjacent normal tissue. $^{**}P < 0.01$ vs. tumor.

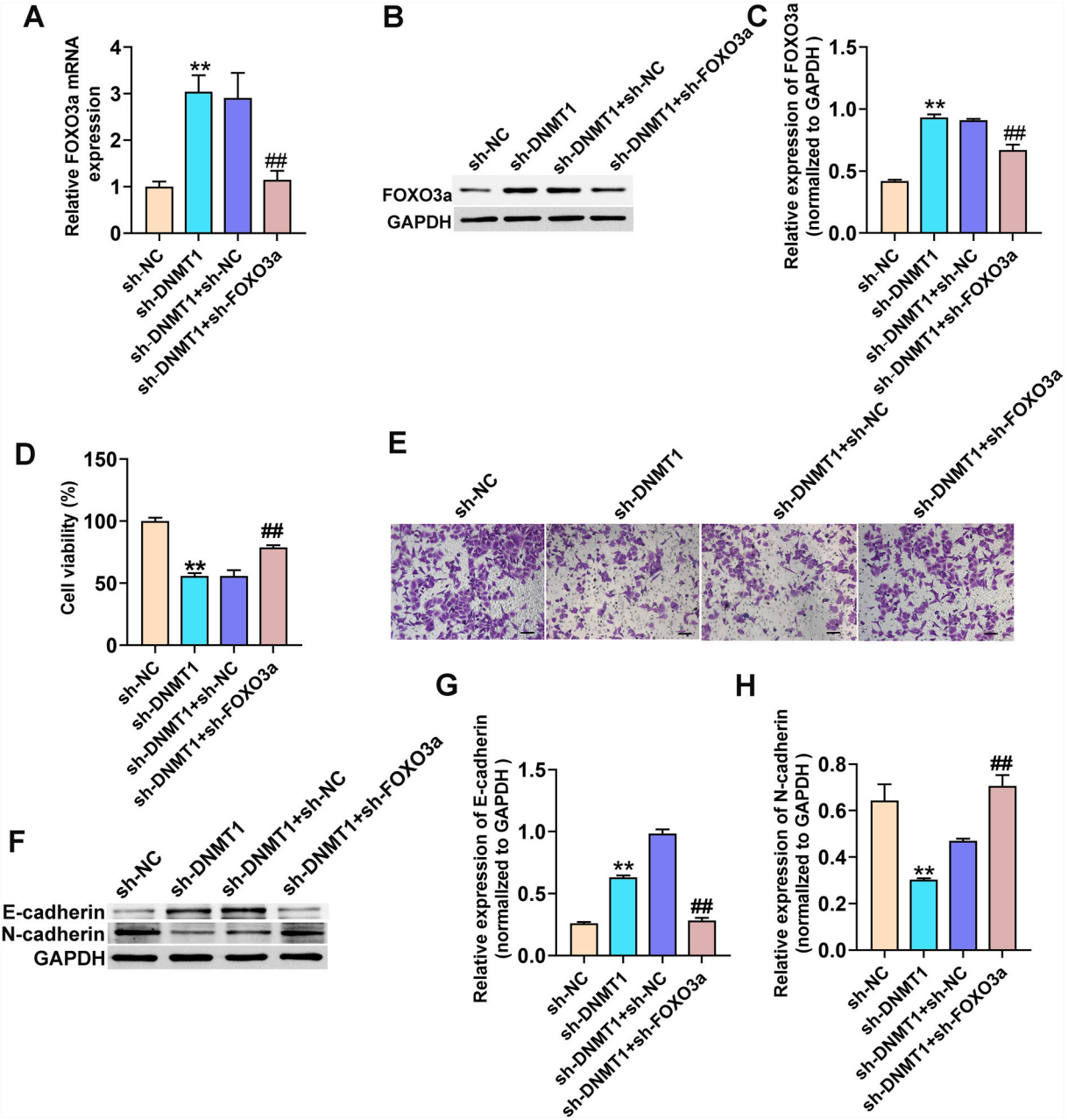

Fig. 4. DNMT1 affects NSCLC cell invasion and EMT by regulating FOXO3a expression. (A) The mRNA expression of FOXO3a was assessed in A549 cells transfected with sh-NC, sh-DNMT1, co-transfected with sh-DNMT1 and sh-NC, and co-transfected with sh-DNMT1 and sh-FOXO3a. (B–C) The protein expression of FOXO3a in A549 cells was assessed after transfection with sh-NC, sh-DNMT1, co-transfection with sh-DNMT1 and sh-NC, and co-transfection with sh-DNMT1 and sh-FOXO3a. (D) The survival rate of A549 cells was determined after infection with sh-NC, sh-DNMT1, co-transfected with sh-DNMT1 and sh-NC, and co-transfected with sh-DNMT1 and sh-FOXO3a. (E) The invasion ability of A549 cells was detected after infection with sh-NC, sh-DNMT1, co-transfected with sh-DNMT1 and sh-NC, and co-transfected with sh-DNMT1 and sh-FOXO3a. Scale bar = 40 μm. (F–H) The protein levels of E-cadherin and N-cadherin in A549 cells were tested using Western blot analysis. GAPDH was used as the internal control. $^{**}P < 0.01$ vs. sh-NC; $^{\#\#}P < 0.01$ vs. sh-DNMT1+sh-NC.

correlation between abnormal DNMT1 levels in NSCLC and m^{6}A modification (Fig. 5A). MeRIP-qPCR assay demonstrated that the level of DNMT1 m^{6}A methylation in A549 cells was significantly higher than in BEAS-2B cells (Fig. 5B). Furthermore, A549 cells exhibited significantly higher expression of the METTL3 protein and mRNA compared to BEAS-2B cells (Fig. 5C–E). This prompted further investigation into the role of METTL3 in regulating m6A methylation of DNMT1 in NSCLC. We first investigated whether *in vitro* interference with METTL3 influences DNMT1 expression and m^{6}A methylation levels in order to further study if METTL3 regulates

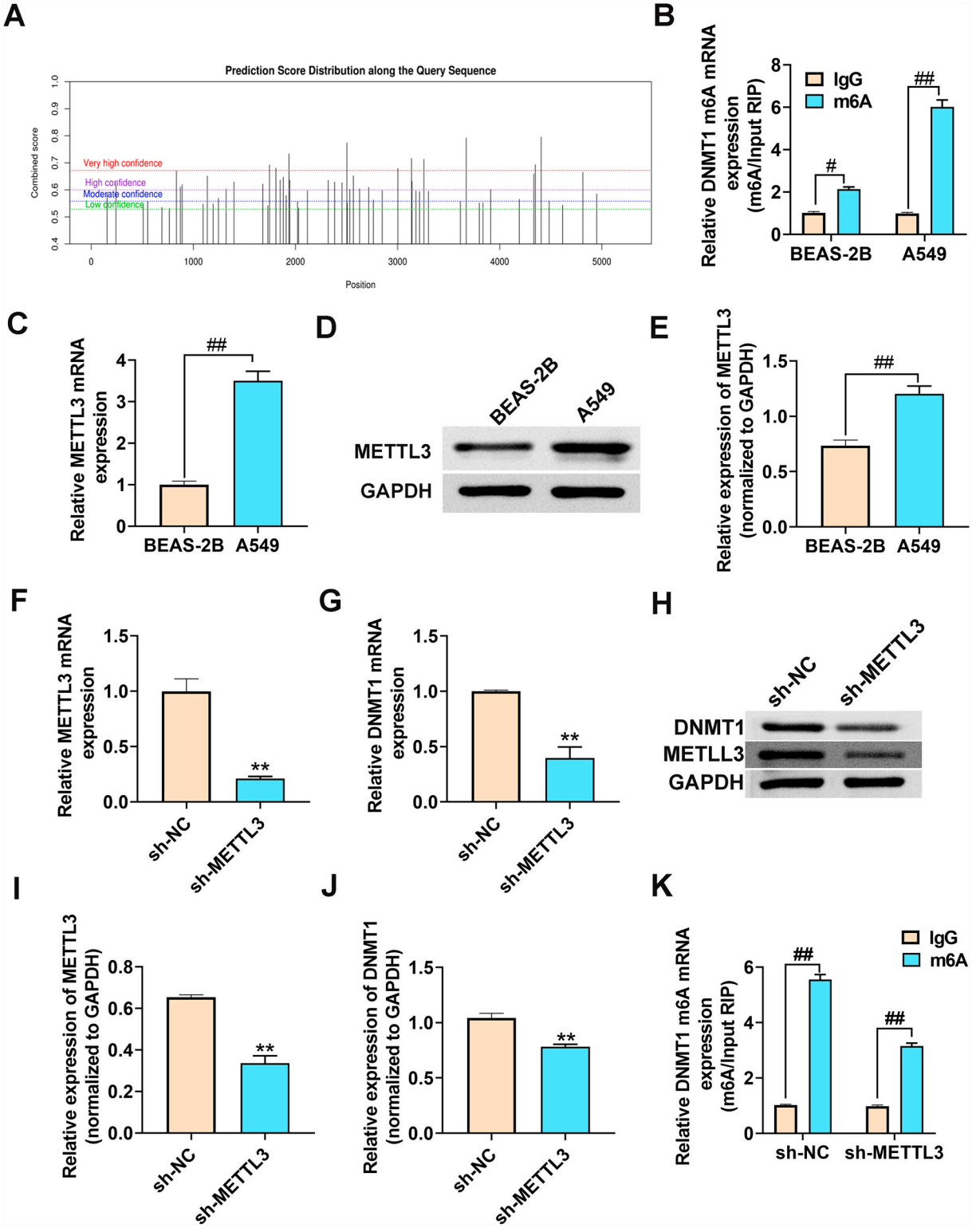

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Fig. 5. The m^6A modification level expression of DNMT1 and METTL3 in NSCLC cells was significantly increased. (A) The SRAMP database (https://www/cuilab.cn/sramp) predicted the m^6A site of DNMT1 mRNA (B) The level of DNMT1 methylation in BEAS-2B cells and A549 cells was measured using MeRIP-qPCR. (C) By using RT-qPCR, the expression level of METTL3 mRNA in BEAS-2B and A549 cells was detected. (D–E) METTL3 protein expression in BEAS-2B cells and A549 cells was detected using Western blot analysis. (F) mRNA expression levels of METTL3 in A549 cells infected with sh-NC and sh-METTL3 were detected using RT-qPCR. (G) mRNA expression levels of DNMT1 in A549 cells infected with sh-NC and sh-METTL3 were detected using RT-qPCR. (H–J) Western blot analysis was used to detect the METTL3 and DNMT1 protein expression levels in A549 cells infected with sh-NC and sh-METTL3. (K) The m6A methylation level of DNMT1 in A549 cells infected with sh-NC and sh-METTL3 was detected using MeRIP-qPCR. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; $^{**}P < 0.01$ vs. sh-NC.

m^6A methylation of DNMT1 in NSCLC. The findings demonstrated that the sh-METTL3 group had considerably greater METTL3 and DNMT1 expression levels compared to the sh-NC group (Fig. 5F–J). Additionally, the m^6A methylation level of sh-METTL3 was lower than that of the sh-NC group (Fig. 5K).

3.5. METTL3 affects NSCLC cell invasion and EMT by regulating the DNMT1/FOXO3a axis

Next, we investigated whether modulation of DNMT1 and METTL3 had an impact on FOXO3a expression and the EMT process in NSCLC cells. First, it was shown that the sh-METTL3+OE-DNMT1 group had higher DNMT1 expression than the sh-METTL3+OE-NC group and that the sh-METTL3 group had significantly lower DNMT1 mRNA and protein levels than the sh-NC group. In contrast, FOXO3a expression in the sh-METTL3+OE-DNMT1 group was lower than in the sh-METTL3+OE-NC group. Additionally, both FOXO3a mRNA and protein levels were significantly higher in the sh-METTL3 group compared to the sh-NC group (Fig. 6A–E).

Notably, we used the CCK-8 test and Transwell assay to investigate the impact of sh-METTL3+OE-DNMT1 in A549 cells. In A549 cells, it was discovered that the sh-METTL3+OE-DNMT1 group exhibited significantly higher rates of cell viability and cell invasion compared to the sh-METTL3+OE-NC group (Fig. 6F–G). Furthermore, compared to the sh-METTL3+OE-NC group, the co-transfection of sh-METTL3 and OE-DNMT1 resulted in a significant reduction in the level of E-cadherin protein and a significant increase in the level of N-cadherin protein (Fig. 6H–J).

3.6. Knockdown of DNMT1 inhibits xenograft tumor growth

To investigate the role of DNMT1 *in vivo*, a NSCLC cancer xenograft model was established. We injected nude mice with DNMT1-shRNA transfected A549 cells, either with or without FOXO3a-shRNA. Tumors in DNMT1 knockdown mice grew more slowly than those in the mice with the control shRNA (Fig. 7A–B). Furthermore, tumor weight was significantly reduced in DNMT1-shRNA transfected nude mice (Fig. 7C). In nude mice NSCLC tissue, DNMT1 knockdown significantly down-regulated DNMT1 and N-cadherin expression, while up-regulating FOXO3a and E-cadherin expression (Fig. 7D–H). Compared to the DNMT1 knockdown group, knockdown both DNMT1 and FOXO3a decreased E-cadherin expression, while increasing N-cadherin expression (Fig. 7D–H).

4. Discussion

NSCLC has one of the lowest survival rates among malignant and highly aggressive cancers [20]. Currently, there are limited therapeutic options available for most patients with advanced NSCLC, apart from chemotherapy and radiation. However, both of these treatments have serious adverse effects [21]. The clinical and biological impact of m^6A has not been thoroughly investigated [22] despite the fact that numerous chemical changes in RNA have been thoroughly verified. Our current focus is on understanding the function and mechanism of METTL3 in NSCLC.

The MethPrimer website predicted a highly methylated site in the FOXO3a promoter region. Indeed, our findings demonstrated that FOXO3a is expressed at low levels in A549 cells. Overexpression of FOXO3a has been shown to suppress cancer cell proliferation, tumorigenic capacity, and invasiveness in experimental studies [23]. First, we observed that whereas DNMT1 expression and the FOXO3a promoter's methylation level were increased, FOXO3a gene expression was down-regulated in A549 cells. *In vivo* and *in vitro* interference of DNMT1 impacts FOXO3a expression by inhibiting the methylation level of the FOXO3a promoter. Next, by controlling FOXO3a expression, DNMT1 influences EMT and NSCLC cell invasion. Then, to determine if FOXO3a down-regulation is connected to promoter methylation, we found that the level of methylation in A549 cells had increased. Additionally, when we administered 5-Aza-dC to A549 cells, we discovered that FOXO3a's mRNA and protein levels had increased. These findings prove that promoter methylation causes down-regulation of FOXO3a in NSCLC.

Our findings for NSCLC are consistent with the hypermethylation of the FOXO3a promoter caused by DNMT1, which has been reported to result in the down-regulation of FOXO3a expression in breast cancer [24]. Another significant practical implication is that DNMT1 can bind to the FOXO3a promoter in a ChIP experiment. To further explore the relationship between DNMT1 and FOXO3a promoter methylation, we found that reducing DNMT1 levels resulted in increased FOXO3a expression in A549 cells. We discovered, through a ChIP assay, that DNMT1 is capable of binding to the FOXO3a promoter. Furthermore, we found, using the MSP, that DNMT1 knockdown can reduce the methylation levels of the FOXO3a promoter.

The EMT and metastasis of breast cancer are prevented by controlling the TWIST-1-mediated miR-10b/CADM2 pathway. Down-regulation of FOXO3a induces the EMT process and promotes the metastasis of pancreatic ductal adenocarcinoma [24,25]. FOXO3a is a prominent transcription factor whose expression is decreased in human tumors. In this research, we demonstrated that the EMT capacity of A549 cells was still reduced even after down-regulating DNMT1 and FOXO3a. Together, these findings show that

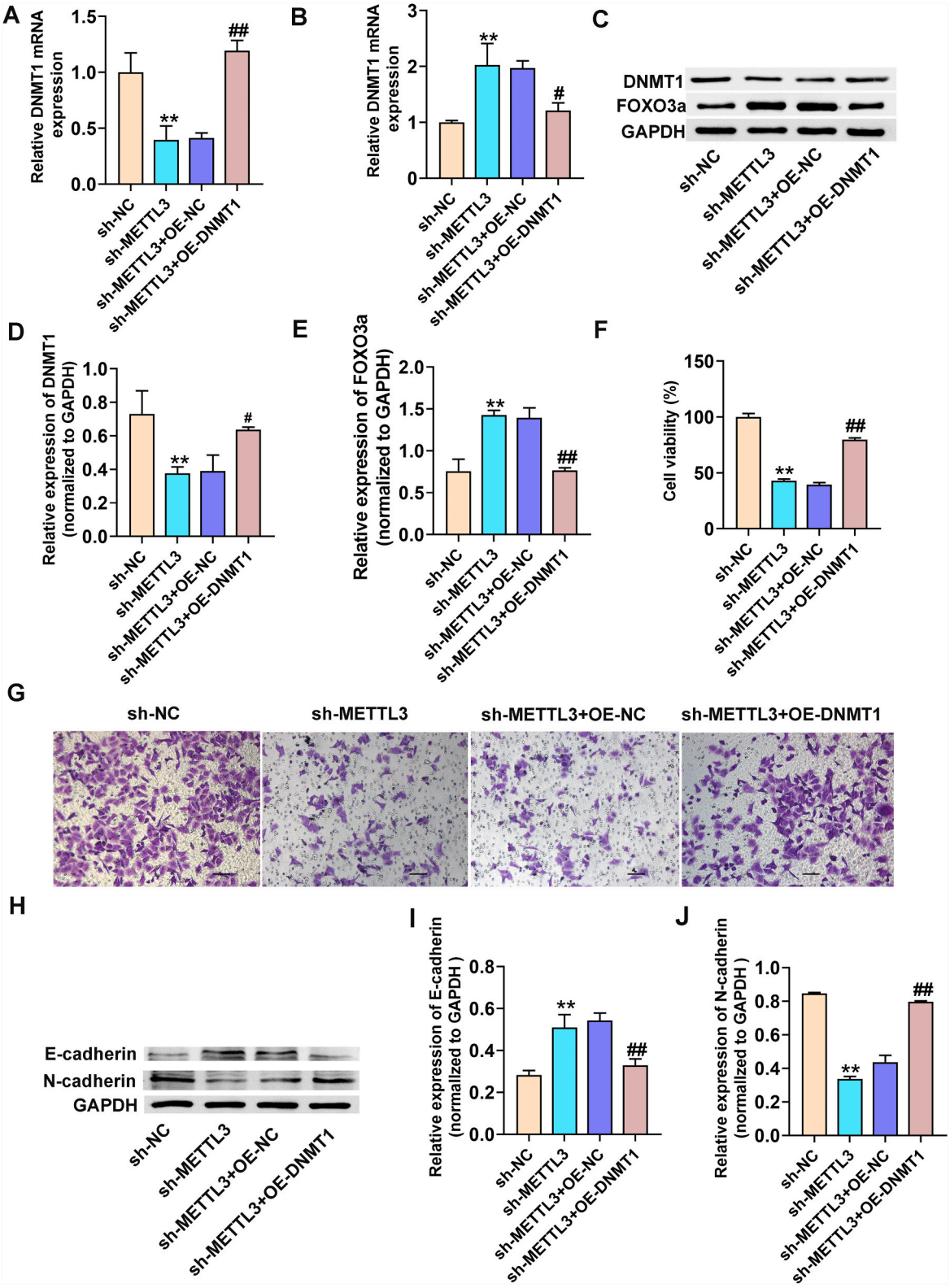

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Fig. 6. METTL3 affects NSCLC cell invasion and EMT by regulating the DNMT1/FOXO3a axis. (A) The mRNA expression levels of DNMT1 in A549 cells infected with sh-NC, sh-METTL3, sh-METTL3+OE + NC, and sh-METTL3+OE DNMT1 were analyzed by RT-qPCR. (B) The mRNA expression levels of FOXO3a were assessed in A549 cells infected with sh-NC, sh-METTL3, sh-METTL3+OE + NC, and sh-METTL3+OE DNMT1 using RT-qPCR. (C–E) The protein levels of DNMT1 and FOXO3a in A549 cells infected with sh-NC, sh-METTL3, sh-METTL3+OE + NC, and sh-METTL3+OE DNMT1 were detected by western blotting. (F) The viability of A549 cells after transfection with sh-METTL3, sh-METTL3+OE + NC, and sh-METTL3+OE-DNMT1 was measured using the CCK-8 assay. (G) The migration of A549 cells was detected using the Transwell assay. Scale bar = 40 μm. (H–J) Western blotting was used to examine the expression of E-cadherin and N-cadherin in A549 cells transfected with sh-NC, sh-METTL3, sh-METTL3+OE + NC, and sh-METTL3+OE-DNMT1. $^{**}P < 0.01$ vs. sh-NC; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. sh-METTL3+OE-NC.

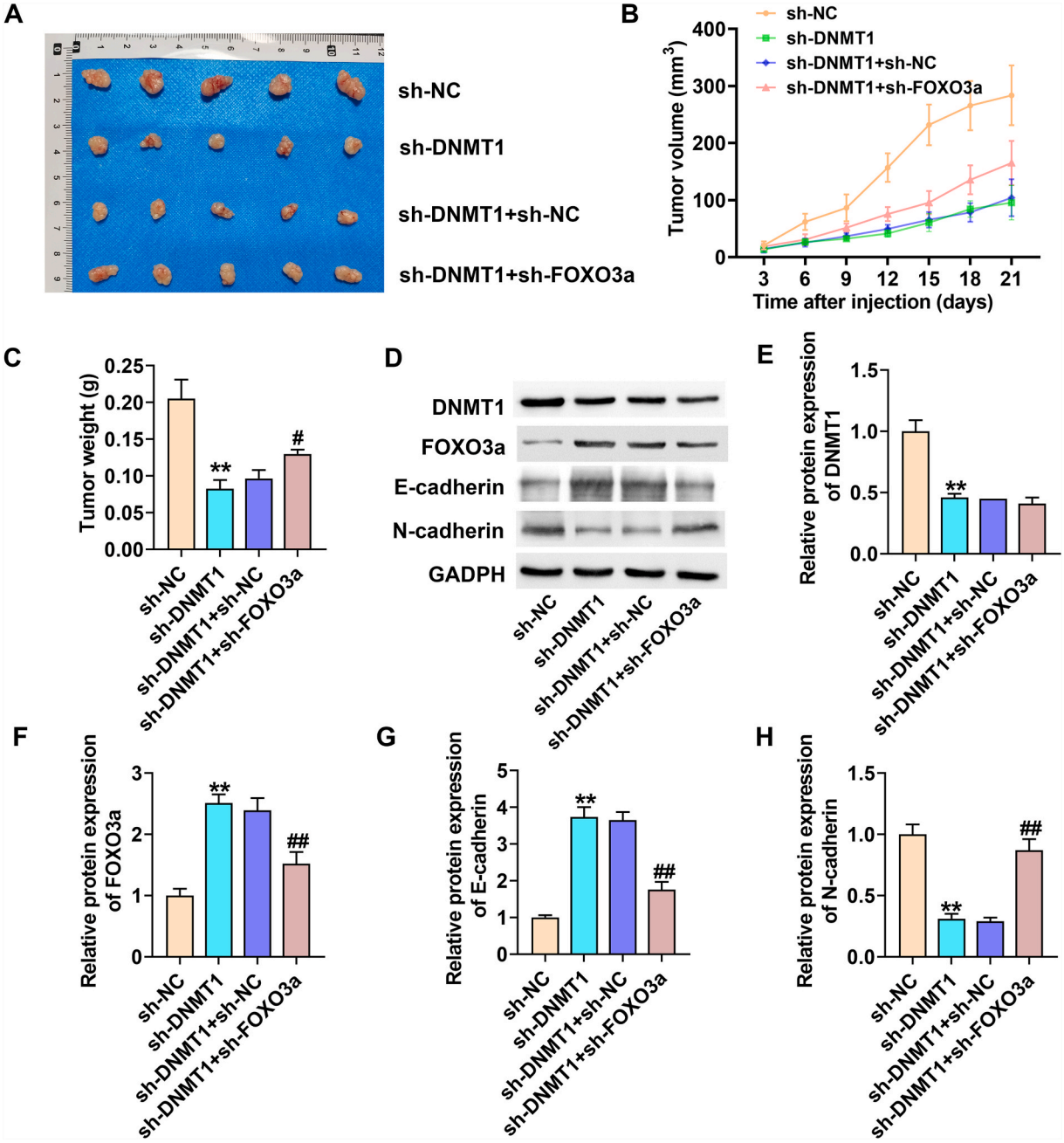

Fig. 7. DNMT1 knockdown suppressed the development of xenograft tumors. A549 cells were infected with sh-DNMT1 with or without sh-FOXO3a. (A) Images of dissected tumors from nude mice. (B) Tumor growth curves in nude mice. (C) Histogram represents mean tumor weight of dissected tumors. (D) Western blot assays were performed to assess protein expression of DNMT1, FOXO3a, E-cadherin and N-cadherin. Quantity analysis DNMT1 (E), FOXO3a (F), E-cadherin (G) and N-cadherin (H) protein expressions. $^{**}P < 0.01$ vs sh-NC; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. sh-DNMT1+sh-NC.

DNMT1 controls FOXO3a expression, influencing NSCLC cell invasion and EMT. The majority of studies have classified FOXO3a proteins as metastasis suppressors because they increase E-cadherin expression while suppressing transcription factors that promote EMT. This, in turn, causes breast cancer cells to lose their invasive phenotype [26]. In addition, FOXO3a can directly bind to β-catenin and regulate its target gene [27]. At the cell membrane of epithelial cells, FOXO3a forms a complex with E-cadherin [28]. These finding suggested that DNMT1-mediated FOXO3A methylation influences EMT through β-catenin. On the other hand, it was found that FOXO3a promoted the invasion of the Hela and MDA-MB-435 cell lines by inducing MMP-13 and Amm-9 [29]. In this study, we discovered that the viability and invasion of NSCLC cells *in vitro* were considerably reduced when FOXO3a was expressed at low levels. To minimize the impacts of cell proliferation on invasion, we added equal number of already treated A549 cells to the upper transwell chamber and utilizing serum-free F–12K media. Therefore, we suggest that FOXO3a plays a role in promoting NSCLC development.

Recent studies have demonstrated the vital and diverse biological roles that m^6A modification plays in the development of different cancers [30]. Methylations such as m^6A, N1-methyladenosine (m^1A), and 5-methyladenosine (m^5C) are the most common RNA alterations [31]. A number of malignancies are influenced by m^6A modifications, although the specific mechanisms by which m^6A-mediated NSCLC progression occurs are not entirely understood. It has been extensively and widely researched in many types of cancer as a major component of the so-called m^6A "writer," METTL3 [32]. METTL3 enhances Bcl-2 expression through m^6A modification, thereby enhancing NSCLC cell survival and migratory capacity [18]. These investigational findings demonstrated that METTL3 was substantially expressed in A549 cells. However, there is limited research on DNMT1's post-transcriptional alteration. Our research revealed that DNMT1 has several m^6A methylation sites, as predicted by SRAMP site prediction. Therefore, we expected that METTL3 might enhance DNMT1's m^6A modification and consequently up-regulate DNMT1 expression. We discovered that A549 cells had higher DNMT1 protein and mRNA levels compared to BEAS-2B cells. Then, in comparison with NSCLC cells, the overall methylation level, DNMT1 m^6A level in A549, and METTL3 expression level were all elevated. Therefore, we believe that METTL3 may also be involved in the modification of DNMT1 m^6A. We discovered the down-regulation of METTL3, which is significant because it could prevent DNMT1 m^6A methylation and reduce DNMT1 expression. Additionally, this work explored the molecular mechanism underlying the role of METTL3 in NSCLC. As a result, the findings revealed that METTL3 modulates m^6A methylation modification to enhance DNMT1 production. This, in turn, leads to decreased FOXO3a expression and increased promoter methylation, thereby assisting the progression of NSCLC.

5. Conclusion

Overall, this study's results suggest that METTL3 up-regulates DNMT1 expression by promoting the m6A alteration of DNMT1. As a result of the down-regulation of FOXO3a expression in NSCLC and the enhanced methylation of the FOXO3a promoter caused by METTL3, NSCLC is more likely to invade and undergo EMT.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics declarations

This study was reviewed and approved by Ethics Committee of Xi 'an International Medical Center Hospital, with ethics approval reference [GJYX-JS-2023-083] for human studies. The ethics approval date is 2023-10-15. Animal experiments were reviewed and approved by Ethics Committee of Xi 'an International Medical Center Hospital, with ethics approval reference [20240122]. All participants/patients provided informed consent to participate in the study.

CRediT authorship contribution statement

Wen-Hai Li: Writing – review & editing, Visualization, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Yi Dang:** Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation. **Liang Zhang:** Software, Methodology, Investigation, Formal analysis, Data curation. **Jin-Cai Zhou:** Methodology, Investigation, Formal analysis, Data curation. **Heng-Yu Zhai:** Methodology, Investigation, Data curation. **Zhao Yang:** Methodology, Investigation, Data curation. **Kai Ma:** Methodology, Investigation. **Zhuang-Zhuang Wang:** Methodology, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28618.

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