Abstract. Methyltransferase-like 3 (METTL3) is an RNA methyltransferase that mediates modification of N6-methyladenosine (m6A), which serves as an oncogene in various types of cancer. The role of m6A modification in the onset and progression of cancer has attracted growing attention. However, the functional and regulatory mechanisms of METTL3 in non-small cell lung cancer (NSCLC) progression are still poorly understood. In the present study, METTL3 expression in NSCLC tissue was analyzed using the Gene Expression Profiling Interactive Analysis database. Western blotting and reverse transcription-quantitative PCR were performed to evaluate the expression of METTL3 in NSCLC tissue and cell lines. Here, knockdown and overexpression of METTL3 notably decreased NSCLC cell viability, apoptosis and migration in vitro and, as well as tumorigenicity in vivo. Expression of METTL3 was upregulated in NSCLC tissue. METTL3 overexpression promoted cell viability and migration in NSCLC, while knockdown of METTL3 yielded the opposite result in vivo and in vitro. METTL3 increased Bcl-2 translation via m6A modification, which increased viability and enhanced migration of NSCLC cells. METTL3 served as an oncogene in NSCLC via METTL3-mediated Bcl-2 mRNA m6A modification, which indicated that targeting METTL3 may be an effective therapeutic strategy for clinical management of NSCLC.

Introduction

Lung cancer is the most common malignancy worldwide and poses a serious threat to human life and health (1). In addition, non-small-cell lung cancer (NSCLC) accounts for ~85% of total lung cancer diagnoses (2). NSCLC is often asymptomatic at the early stage of the disease and most patients are diagnosed when the disease has progressed to the intermediate or late stage (2,3). Although an increasing number of diagnostic and therapeutic strategies have been developed, the overall survival rate remains unsatisfactory (4). Some patients still die due to distant metastasis and drug resistance (5,6). Thus, it is key to investigate the potential molecular mechanisms of NSCLC and to identify novel treatment approaches to improve outcomes in patients with NSCLC.

RNA N6-methyladenosine (m6A) modification is the most abundant conserved post-translational modification in eukaryotic organisms (7). It primarily regulates gene expression, mRNA stability, alternative splicing, translation efficiency and primary microRNA (miRNA) processing (8,9). The dynamic modification of m6A is regulated by two types of catalytic proteins (m6A ‘writers’ and ‘erasers’) (10). The ‘writers’ primarily include methyltransferase-like (METTL)3, METTL14 and Wilms tumor 1 associated protein (11,12). ‘Erasers’, such as alkylation repair homolog protein 5 and fat mass and obesity-associated protein, serve as demethylases to reverse methylation (13). A previous study demonstrated that m6A modulators exhibit various biological functions in different processes, such as embryonic growth, stem cell differentiation, circadian rhythm and tumor progression (14).

METTL3 is a core methyltransferase for m6A modification (15). Certain studies have reported that METTL3 serves an oncogenic role in gastric, colorectal and bladder cancer, as well as hepatocellular carcinoma (15-18). By contrast, a recent study showed that METTL3 is a tumor suppressor in kidney cancer (19). Nevertheless, the biological function of METTL3 and its underlying regulatory mechanisms in NSCLC have not been sufficiently described.

The aim of the present study was to investigate METTL3 expression in NSCLC cells and human NSCLC tissue and to determine the role of METTL3 in NSCLC progression both in vitro and in vivo. The results of the present study may provide a novel therapeutic target for treatment of NSCLC.

Materials and methods

Correspondence to: Professor Chengxue Dang, Department of Oncology, The First Affiliated Hospital of Medical College, Xi’an Jiaotong University, 277 West Yanta Road, Xi’an, Shaanxi 710061, P.R. China
E-mail: dangchengxue@aliyun.com

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Patients and specimen collection. A total of 77 NSCLC tissue samples and paired adjacent normal tissue samples (distance, ≥3 cm) were obtained from patients undergoing surgery from...
May 2018 to January 2020 at the First Affiliated Hospital of Xinxiang Medical University (Xinxiang, China). Of these patients, 44 were male (mean age, 60.22 years) and 33 were female (mean age, 58.6 years). The histological type of the tumors was assessed using the World Health Organization Classification (20) by two independent pathologists. Ethics approval was granted by the Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University (approval no. KN201808002). Oral consent was obtained from patients. All experiments were performed in accordance with approved guidelines and regulations (21).

Cell culture. Human NSCLC cell lines (A549, PC9, H1299, H1975 and HCC827) and normal pulmonary epithelial cells (BEAS-2B) were obtained from the American Type Culture Collection. A549, PC9, H1299, H1975 and HCC827 cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.). BEAS-2B cells were cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.). All media were supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and all cells were incubated at 37°C with 5% CO2.

Establishment of stable cell lines. Stable cell lines were constructed using lentivirus system. The plasmids used to make stably overexpressed/knockdown cell lines were based on the pcDNA3/Flag-METTL3 plasmid (Addgene; cat. no. 53739) or short hairpin (sh)RNA-METTL3 (GenePharma Co., Ltd.). To generate lentiviruses particles, H1299 or H1975 cells were co-transfected with plasmids together with Trans-lentiviral packaging plasmids using Trans-lentiviral Packaging kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Lentivirus particles were harvested at 48 h post transfection and concentrated using Lenti-X concentrator according to manufacturer instruction of the chamber was filled with 600 µl RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.). The lower portion of the chamber was filled with 600 µl RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 20% FBS (Gibco; Thermo Fisher Scientific, Inc.) and all cells were incubated at 37°C with 5% CO2.

Cell viability assay. Cells were seeded in 96-well plates at a density of 2,000 cells/well. Cells were incubated at 37°C in an incubator with 5% CO2 for 0, 24, 48 and 72 h. Cell Counting Kit-8 (CCK-8; Sigma-Aldrich; Merck KGaA) was used to measure cell viability according to the manufacturer's instructions. The absorbance was measured at a wavelength of 450 nm using a plate reader (Bio-Rad Laboratories, Inc.).

Apoptosis. Apoptosis was measured using flow cytometry with a FITC-Annexin V/PI detection kit (Wanleibio Co., Ltd.). A total of 1x10^4 cells per well was seeded in 6-well plates and cells were then harvested after culturing for 48 h at 37°C. Afterwards, the cells were stained in the dark with FITC-Annexin V and PI at room temperature for 15 min. Subsequently, the percentages of apoptotic cells were measured using FACS flow cytometry (BD Biosciences) and analyzed using FlowJo 8.6.3 (Tree Star, Inc.).

Cell migration assay. Cell migration assays were performed using a cell migration assay kit (Corning, Inc.) and Transwell inserts. Briefly, 2x10^5 cells were seeded in the upper chamber of a Boyden chamber (without serum in the RPMI-1640 medium, Invitrogen; Thermo Fisher Scientific, Inc.). The lower portion of the chamber was filled with 600 µl RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 20% FBS (Gibco; Thermo Fisher Scientific, Inc.). Following incubation for 24 h at 37°C, migrated cells in the lower chambers were fixed with 4% paraformaldehyde for 15 min at room temperature and stained with 0.05% crystal violet (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature. Cells were counted in five randomly selected fields of view under a light microscope (Leica GmbH DMI4000B) at 200x magnification.

m6A qPCR. m6A qPCR was performed as reported previously (23) to assess the relative abundance of the selected mRNA in m6A antibody immunoprecipitation (IP) and input samples. Isolated m6A-IP RNA was quantified by qPCR. The primers used were as follows: Bcl-2 forward, 5'-ATC GCC AAA TCA TCA CAT-3'; and reverse, 5'-GAA AAC GGA GAA ACC AAG-3'.

m6A sequencing (m6A-seq). Total RNA was isolated and purified using PolyATract mRNA Isolation Systems kit (cat. no. Z5310; Promega Corporation). m6A-seq was performed as previously described (24). The NEBNext Ultra Directional RNA Library Prep kit (cat. no. E7420L; Illumina, Inc.) was used for library construction. Clustered libraries were sequenced with an Illumina HiSeq 4000 (Illumina, Inc.) by Novogene Co., Ltd.
Western blotting. Total protein was extracted from cells using RIPA buffer containing protease and phosphatase inhibitor cocktail (both from Sigma-Aldrich; Merck KGaA). The protein concentration was determined with a BCA Protein Assay kit (Beyotime Institute of Biotechnology). Cellular proteins were separated by 10% SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk and 0.1% Tween-20 at room temperature for 2 h. Membranes were probed at 4°C overnight with primary antibodies against METTL3 (1:2,000; cat. no. 86132S; Cell Signaling Technology, Inc.), Bcl-2 (1:1,000; cat. no. ab32124; Abcam) and GAPDH (1:5,000; cat. no. 5174S; Cell Signaling Technology, Inc.). After washing in PBST (0.5% Tween-20). Three times, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1,000; cat. no. BA1070; Wuhan Boster Biological Technology, Ltd.) at room temperature for 1 h. The signal was visualized using ECL western blotting detection reagents (Tanon Science and Technology Co., Ltd.). Protein expression levels were quantified using ImageJ software (version 1.50; National Institutes of Health).

Experimental mouse techniques. A total of 15 female BALB/c nude mice (age, 6 weeks; weight, 18-20 g) were purchased from the Model Animal Center at Nanjing University (Nanjing, China) and raised under pathogen-free conditions in the Xinxiang Medical University Animal Center (Xinxiang, China). The mice were housed with filtered air, 12 h light/dark cycle, constant temperature (25°C), relative humidity (50±5%) and free access to food and water. In order to establish a human NSCLC xenograft model, 5x10⁶ H1299, sh-METTL3-H1299 and METTL3 stably overexpressed H1299 cells (2x10⁶ per mouse) were subcutaneously injected into mice. Tumor growth was observed daily. Tumor volume was calculated as follows: 0.5x (length x width²). At 24 days post-inoculation, the maximum diameter exhibited by a single subcutaneous tumor was 15 mm and mice were anesthetized by intraperitoneal administration of sodium pentobarbital (50 mg/kg), then sacrificed by cervical dislocation. Tumors were harvested and weighed for histological analysis. Lungs were extracted and fixed for 24 h at room temperature using 10% (v/v) neutral-buffered formalin and transferred to 70% ethanol until embedding in paraffin then sectioned at 3 μm. For hematoxylin-eosin staining, sections were incubated with hematoxylin for 2 min and eosin for 1 min. The experimental procedures were approved by the Laboratory Animal Care Committee at Xinxiang Medical College (approval no. XXMU-2020-146367).

Immunohistochemistry (IHC) assay. Tissue sections were incubated with primary antibodies against METTL3 (1:50; cat. no. 86132S; Cell Signaling Technology, Inc.), Bcl-2 (1:50; cat. no. ab32124; Abcam) and Ki67 (1:100; cat. no. ab16667; Abcam) at 4°C overnight. Following washing with PBST containing 0.05% Tween-20, the sections were incubated with HRP-conjugated anti-rabbit secondary antibody (1:5,000; cat. no. BM3894; Wuhan Boster Biological Technology, Ltd.) at room temperature for 1 h. The sections were developed with 0.05% 3-diaminobenzidine tetrahydrochloride at room temperature for 10 sec, which was followed by counterstaining with 10% Mayer's hematoxylin for 4 min at room temperature. IHC results were analyzed by two experienced pathologists. A total of five random fields under a light microscope (Leica GmbH DMI4000B) at 200x magnification were chosen to calculate the percentage of positively stained cells vs. the total number of tumor cells. The staining proportion of the positive cells was divided into four groups as follows: -, 0 positive cells observed; +, <30% vs. positive tumor cells observed; ++, 30-60% vs. positive tumor cells observed; and +++: >60% vs. positive tumor cells observed. The - and + groups were classed as METTL3-low expression group, while the ++ and +++ groups were classed as METTL3-high expression group for subsequent analysis. Low- and high-expression groups were combined following scoring and analyzed by Fisher's exact t-test.

Bioinformatics analysis. The Gene Expression Profiling Interactive Analysis (GEPIA) database (gepia.cancer-pku.cn/) was used to evaluate the mRNA expression levels of METTL3 in NSCLC and normal tissue. Log rank test was used for Kaplan-Meier analysis. The Kaplan-Meier plotting tool (kmplot.com/analysis/) was used to evaluate the relapse-free survival of patients with NSCLC expressing low or high levels of METTL3. The association between METTL3 and Bcl-2 mRNA expression levels in NSCLC was analyzed using GEPIA database (gepia.cancer-pku.cn/) and Pearson's correlation coefficient.

Statistical analysis. A total of three independent repeats of experiments were performed. All data are shown as the mean ± SD. Data were analyzed using SPSS software 22.0 (IBM Corp.). Associations between two groups were evaluated using Chi² test and Pearson's correlation analysis. Comparisons between two groups were performed using Student's unpaired two-tailed or paired t-test. Multiple groups were analyzed using one-way ANOVA followed by Bonferroni's post hoc test for pairwise comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

METTL3 expression is elevated in NSCLC tissue and cells. First, it was determined that the expression of METTL3 was upregulated in the NSCLC cohort using bioinformatics tools (gepia.cancer-pku.cn/; Fig. 1A). In addition, RT-qPCR analysis of METTL3 mRNA expression in samples from patients with NSCLC showed that the expression of METTL3 was significantly higher in NSCLC tumor samples than in normal paracancerous tissue (Fig. 1B). m6A quantitative analysis showed that the m6A content level was significantly higher in tumor than in normal paracancerous tissue (Fig. 1C). Western blot and IHC analysis showed that the protein expression of METTL3 was increased in NSCLC compared with normal paracancerous tissue (Fig. 1D and E). Clinical and pathological data are shown in Fig. 1F. A significant positive association between high METTL3 expression and tumor differentiation, tumor stage and metastasis was observed. These results indicated that METTL3 served a significant role in NSCLC development and progression. Consistently, METTL3 expression was increased in the NSCLC cell lines compared with
the normal human lung epithelial cell line BEAS-2B (Fig. 1G). Kaplan-Meier survival analysis indicated that patients with tumors with high METTL3 expression had a poor survival rate and outcome (Fig. 1H).

**METTL3 knockdown inhibits NSCLC cell viability and migration in vitro.** In order to investigate the functional role of METTL3 in NSCLC, METTL3 was knocked down using three independent shRNAs (shRNA-1, shRNA-2 and shRNA-3) in an NSCLC cell line (H1299). Efficient knockdown of METTL3 was confirmed by RT-qPCR (Fig. 2A) and western blotting (Fig. 2B). The CCK-8 assay results showed significantly decreased viability of NSCLC cell lines (H1299, H1975) after silencing METTL3 (Fig. 2C). Moreover, flow cytometry assay revealed that METTL3 silencing accelerated apoptosis in NSCLC cell lines (H1299, H1975; Fig. 2D). Migration assay revealed that METTL3 silencing repressed migration (Fig. 2E). m6A-qPCR validation showed that the percentage of m6A content was significantly decreased when METTL3 was knocked down in H1299 and H1975 cell lines (Fig. 2F).
Collectively, these results indicated that silencing METTL3 suppressed viability and migration and promoted apoptosis in NSCLC cell lines. METTL3 overexpression promotes NSCLC cell viability and migration in vitro. Next, stable METTL3-overexpressing NSCLC cell lines (H1299 and H1975) were constructed by
lentiviral infection. Upregulation of METTL3 expression was confirmed by RT-qPCR (Fig. 3A) and western blotting (Fig. 3B). METTL3 overexpression significantly promoted NSCLC cell line (H1299 and H1975) viability, as determined by CCK-8 assay (Fig. 3C). Moreover, flow cytometry revealed that overexpression of METTL3 inhibited the apoptosis of NSCLC cell lines (H1299 and H1975; Fig. 3D). Furthermore, METTL3 overexpression significantly increased migration of NSCLC cell lines in the Transwell assay (Fig. 3E). Quantitative analysis of m6A showed that METTL3 overexpression...
increased m6A content (Fig. 3F). Based on these results, it was concluded that METTL3 overexpression promoted NSCLC cell viability and migration.

**Bcl-2 is a downstream target of METTL3.** A previous study reported that Bcl-2 may be mediated by METTL3 in breast cancer (21). In order to identify the underlying molecular mechanisms by which METTL3 promotes NSCLC progression, m6A-seq was performed. The results showed that the GGAC motif was highly enriched within m6A sites in the immunopurified RNA (Fig. 4A). In order to verify that METTL3 targets Bcl-2 mRNA for m6A modification, ethylated RNA immunoprecipitation qPCR was performed to validate the m6A-seq data. The results showed that METTL3 knockdown significantly decreased m6A levels of Bcl-2 mRNA (Fig. 4B). RT-qPCR analysis showed that Bcl-2 expression was significantly decreased at the mRNA level following METTL3 knockdown in H1299 and H1975 cells (Fig. 4C). Bcl-2 protein expression was significantly downregulated in METTL3-knockdown H1299 and H1975 cells (Fig. 4D).

Then, the online bioinformatics tool GEPIA database (gepia.cancer-pku.cn/) was used for correlation analysis and our results showed that the METTL3 expression level also showed a positive correlation with Bcl-2 in tumors from individuals with NSCLC (Fig. 4E). Thus, it was concluded that Bcl-2 was a target of METTL3.

**METTL3 facilitates NSCLC tumorigenesis by enhancing expression of Bcl-2 in vivo.** In order to confirm the potential contribution of METTL3 to tumor progression in vivo, BALB/c nude mice were subcutaneously injected with H1299 cells with METTL3 overexpression or knockdown to develop an NSCLC xenograft model. After 24 days, the tumors were removed, photographed and weighed (Fig. 5A and B). Tumors arising from cells with METTL3 overexpression were significantly larger and heavier than those arising from control cells (Fig. 5C). By contrast, knockdown of METTL3 significantly repressed tumor growth (volume) compared with that of tumors arising from control cells (Fig. 5C). Hematoxylin-eosin staining indicated that knockdown of METTL3 decreased the number of metastatic nodules, while overexpression of METTL3 increased the number of lung metastatic nodules.
compared with control (Fig. 5D). In order to confirm whether METTL3 promoted tumor growth in vivo by regulating the expression of Bcl-2, IHC staining and western blot analysis were performed. The results indicated that METTL3 overexpression increased Bcl-2 expression and METTL3 knockdown inhibited Bcl-2 expression (Fig. 5E-F). Collectively, these results suggested that METTL3-mediated m6A modification promoted NSCLC progression by enhancing expression of Bcl-2 in vivo.

Discussion

Recently, the role of m6A modification in mRNA translation has emerged as a hot spot in the field of epigenetics (25). Emerging evidence indicates that m6A modification serves important and diverse biological functions in multiple cellular processes, such as RNA synthesis, processing, translation and metabolism (26,27). m6A modification regulates the tumorigenesis of cancers (28,29). For example, in breast cancer tumorigenesis, m6A demethylase ALKBH5 mediates m6A modification in the 3'-UTR of NANOG mRNA (30). Therefore, more detailed work is needed to define the role and underlying mechanisms of m6A modification in certain types of cancer.

Certain studies have demonstrated that METTL3 serves a regulatory role in a variety of cancercytes, such as breast, bladder and lung cancer, as well as hepatocellular carcinoma (31-35). For example, METTL3 can act as an oncogene in bladder tumorigenesis (36); however, METTL3 and METTL14 also serve as tumor suppressors in hepatocellular carcinoma (37,38). Certain studies have reported that METTL3 contributes to transforming...
growth factor-β-induced epithelial-mesenchymal transition of lung cancer cells (39). An additional study revealed that there is a significant correlation between METTL3 expression and the occurrence of chronic obstructive pulmonary disease (40). However, the role of METTL3-mediated m6A modification in NSCLC initiation and progression is not fully known. The present study demonstrated that METTL3 expression was significantly upregulated in NSCLC. The role of METTL3 in promoting NSCLC progression was demonstrated both in vitro and in vivo. Bcl-2 was shown to be a direct target of METTL3-mediated m6A modification in NSCLC. Finally, the present findings shed new light on m6A methylation-mediated Bcl-2 overexpression during progression of NSCLC and identified that METTL3 serves an oncogenic role as an m6A ‘writer’ in NSCLC. Thus, METTL3 is a promising therapeutic target for NSCLC treatment.

A recent study indicated that METTL3 regulates expression of various mRNAs in bladder cancer, including MYC, NF-κB and AF4/FMR2 family member 4 (41). Hence, it was hypothesized that METTL3 may promote the translational efficiency of Bcl-2 mRNA to regulate NSCLC progression. Similarly, in breast cancer, METTL3-mediated m6A modification of Bcl-2 promotes its expression (27). Furthermore, METTL3 and Bcl-2 expression levels were positively correlated. Certain studies have revealed that METTL3 overexpression directly mediates m6A methylation and inhibits apoptosis in various types of cancer (42,43). For example, overexpression of Bcl-2 blocks Adriamycin-induced apoptosis in bladder cancer cells (44). Another report revealed that high Bcl-2 expression is associated with recurrence and poor survival in gastric cancer (45-46).

Taken together, the results of the present study highlight the critical role of METTL3 in NSCLC progression. METTL3 regulated cellular growth, survival and migration in NSCLC. METTL3 promoted NSCLC progression by modulating the level of Bcl-2. This finding may contribute to understanding of the oncogenic role of METTL3, which may be an effective therapeutic target for NSCLC.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Sequence Read Archive repository, accession no. PRJNA721237.

Authors’ contributions

The project was designed and conceived by YZ and CD. SL, YZ and TZ analyzed the data. YZ wrote the manuscript. YZ and CD guided the study and revised the manuscript. YZ, SL, TZ and CD confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Ethics approval was granted by the Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University (approval no. KN201808002). Oral consent was obtained from patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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