Methyltransferase 3, N6-adenosine-methyltransferase complex catalytic subunit-induced long intergenic non-protein coding RNA 1833 N6-methyladenosine methylation promotes the non-small cell lung cancer progression via regulating heterogeneous nuclear ribonucleoprotein A2/B1 expression

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ABSTRACT
Long intergenic non-protein coding RNA 1833 (LINC01833) exhibits elevated expression in the non-small cell lung cancer (NSCLC) tissues, while its molecular mechanism in NSCLC progression remains elusive. Herein, the proliferation, migration, invasion as well as apoptosis of NSCLC cells were assessed. The potential N6-methyladenosine (m6A) modification site was predicted by the m6aVar tool. RNA pulldown and m6A-specific immunoprecipitation assays were used to detect the interaction between LINC01833 and methyltransferase 3, N6-adenosine-methyltransferase complex catalytic subunit (METTL3). RNA pull-down together with mass spectrometry were performed to assess the binding relationship between LINC01833 and heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1) in NSCLC. Tumor xenograft mice model was established, and the tumor size and weight were measured. The results demonstrated that LINC01833 expression was elevated in NSCLC samples. Overexpression of LINC01833 promoted proliferative, migratory, and invasive abilities and inhibited HCC827 cell apoptosis. LINC01833 knockdown inhibited tumor growth in mice. LINC01833 is further demonstrated to be modulated by METTL3, which is highly expressed in NSCLC samples. In addition, RNA pulldown and m6A-specific immunoprecipitation assays indicated that LINC01833 might form a complex with HNRNPA2B1. In conclusion, m6A transferase METTL3-induced LINC01833 m6A methylation promotes NSCLC progression through modulating HNRNPA2B1 expression. Our findings indicated that LINC01833 might be a therapeutic target for NSCLC.

1. Introduction
Lung cancer is the most common malignancy worldwide, and its morbidity and mortality rank first in malignancies, of which non-small cell lung cancer (NSCLC) accounts for approximately 80% [1]. Since NSCLC has an insidious onset, the symptoms are not specific at the beginning. At present, the main treatment methods for lung cancer include surgical resection, chemotherapy, radiation therapy, targeted therapy, and immunotherapy that can greatly extend the survival time of patients [1,2]. However, because the pathogenesis of lung cancer is unknown and preventive and therapeutic methods are lacked, its morbidity...
and mortality are still high [3]. Therefore, exploring the mechanism of NSCLC and mining key regulatory factors are of great significance for the targeted therapy of NSCLC.

LncRNAs have over 200 nucleotides in length and lack the frame of open reading, with little or without protein coding ability [4,5]. It has been revealed that LncRNAs play vital roles in cell physiology and pathological activities and involve in occurrence and development of tumors [6,7]. Multiple studies have illustrated that LncRNAs exert vital roles in the NSCLC progression [8–10]. Liang et al. indicated that small nucleolar RNA host gene 10 is downregulated in NSCLC tissues and it predicts poor survival of patients with NSCLC [11]. Chen et al. found that competing endogenous LncRNA 2 for microRNA let-7b was highly expressed in NSCLC tissues and enhanced gefitinib-resistance in NSCLC by down-regulating miRNA-621 [12]. In addition, several LncRNAs were revealed as important biomarkers in NSCLC [13,14], such as long intergenic non-protein coding RNA 504 and long intergenic non-protein coding RNA 691 [15,16]. Recently, Yu et al successfully identified five survival-related LncRNAs using transcriptome profiling analysis of NSCLC from TCGA database [17]. Among them, the expression of long intergenic non-protein coding RNA 1833 (LINC01833) showed the most significant upregulation in tumors compared with normal group, suggesting that LINC01833 might act as an oncogene in NSCLC. However, the regulatory mechanism of LINC01833 remains unclear.

N6-methyladenosine (m6A) methylation widely exists in mammals and regulates gene expression after transcription without changing the base sequence [18]. m6A methylation affects tumor occurrence and development via regulation on proto-oncogene and tumor suppressor gene expression at the level of epigenetic modification through methyltransferase and demethyltransferase [19,20]. Several genes such as yes-associated protein and RNA component of mitochondrial RNA processing endoribonuclease were regulated by m6A methylation that participated in the development of lung cancer [21,22]. In addition, methyltransferase 3, n6-adenosine-methyltransferase complex catalytic subunit (METTL3) affects the tumor formation by regulating the m6A modification in LncRNAs [23–25].

Heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1) is a protein that ubiquitously participates in RNA-binding and pre-RNA processing, regulating cancer cell metabolism, proliferation, migration, invasion, and apoptosis [26–30]. HNRNPA2B1 shows high expression in lung cancers [31,32] and serves as a m6A regulator [33,34].

Based on bioinformatics analysis, there are m6A modification sites on LINC01833. We therefore hypothesized that METTL3 mediates m6A modification in LINC01833 to regulate NSCLC progression. Detailed functions of METTL3 and LINC01833 in NSCLC were further explored. Moreover, we provided an insight into the downstream molecular mechanism of LINC01833 in NSCLC.

2. Materials and methods

2.1 Bioinformatics analysis

The online tool m6aVar (http://m6avar.renlab.org/) was used to reveal the m6A modification sites on LINC01833. RNA binding proteins of LINC01833 were obtained from online tool Starbase (https://starbase.sysu.edu.cn/), which were listed based on the number of CLIP-seq experiments. HNRNPA2B1 was selected since it ranks the first.

2.2 NSCLC Specimen and cell lines

A cohort of 30 paired NSCLC specimens and adjacent tissues were obtained from Shanxi Provincial Cancer Hospital between December 2019 to December 2020. These specimens were immediately frozen and stored in liquid nitrogen. This study was approved by the Research Ethics Committee of Shanxi Provincial Cancer Hospital and informed consent was obtained from all patients. A copy of the ethical approval was provided in Supplementary material 1. NSCLC cell lines HCC827, NCI-H1299, A549 and NCI-H1650 and the normal lung epithelial cell BEAS-2B were obtained from COBIOER (Nanjing, China) and were cultured in Roswell Park Memorial Institute (RPMI) 1640 basic medium supplemented with 10% fetal bovine serum (FBS) in a humidified with 5% CO₂ at 37°C.
2.3 Plasmid construction and cell transfection

The cDNA of LINC01833 or METTL3 was synthesized and cloned into the expression vector pcDNA3.1 (GenePharma, Shanghai, China). shRNA plasmids were designed by GenePharma to knock down LINC01833 or METTL3. The pcDNA3.1 vectors and shRNA plasmids were transfected into NSCLC cells HCC827 using RNAiMAX following the manufacturer’s manual after the cell confluence reached 70–90%. Cells were collected at 48 h post-transfection.

2.4 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from tissue samples or cells using TRIZOL regent and the concentrations of total RNA were analyzed by Nanodrop. Reverse transcription of total RNA was performed using the PrimeScript RT Reagent Kit (Takara). The quantitative polymerase chain reaction (qPCR) was performed by ABI7500 using the SYBR Premix Ex Taq (Takara). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The relative gene expression levels were calculated using the 2^-ΔΔCT method. The primers sequences are as follows: LINC01833-F, 5'-CATTACAGGCGATCA CCCAT-3', LINC01833-R, 5'-GACAAACCCAGGT ACCT-3'; HNRNPA2B1-F, 5'-CCAGGGACATTT ACTCAGACCAAT-3', HNRNPA2B1-R, 5'-GGAC ACCTACCTTATTACCTCCC-3'; U6-F, 5'-TACCT ACCCAGGACCAGG-3'; U6-R, 5'-CCATAGAC CTGGAAGCT-3'; GAPDH-F, 5'-GGACCTGCATT ACTCCGAATCT-3', GAPDH-R, 5'-CGGGCCCATAT TACATACT-3'.

2.5 Western blot

Proteins were isolated by RIPA buffer (Beyotime) and determined using a BCA protein kit (Beyotime). Thirty µg protein was loaded into a 10% sodium dodecyl sulfate polyacrylamide gel and then electrophoresed, transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% fat-free milk for 2 h at room temperature and incubated with primary antibodies against METTL3 and HNRNPA2B1 overnight at 4°C. Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated immunoglobulin G secondary antibody for 2 h at room temperature. Enhanced chemiluminescence reagent was applied to the membranes for visualization. Protein bands were analyzed by Quantity One software version 4.62 (Bio-Rad). Original Western blot images were provided in Supplementary material 2.

2.6 Cell counting kit-8 (CCK-8) assay

The cells with different transfections were incubated at 37°C in a 5% CO2 incubator for 24 h and then were harvested, resuspended in a culture medium, and seeded into 96-well plates at a density of 2 × 10^3 cells/well. Cell proliferation was assessed by incubating cells with 10 µl of CCK-8 solution for 0, 24, 48, and 72 h at 37°C for 2 h. The optical density (OD) was measured at 450 nm using a Tecan microplate reader.

2.7 Flow cytometric assays

For cell apoptosis analysis, cells were harvested at 48 h after transfection. Propidium iodide (PI) and fluorescein isothiocyanate (FITC)-Annexin V were used to stain the cells in the dark. Cell apoptosis was measured by a flow cytometer (FACScan; BD Biosciences). Original flow cytometry data were provided in Supplementary material 3.

2.8 Cell migration and invasion assays

Transfected cells were collected and suspended to a density of 1 × 10^5 cells/ml. For cell migration assay, upper chamber was equipped with 8-µm porous membranes (BD Biosciences) and added with 100 µl of the suspension. Six hundred µl of complete culture medium was added to each lower chamber to induce migration. The non-migrated cells were removed with a cotton swab after incubation for 24 h at 37°C with 5% CO2 whereas the migrated cells were fixed with 4% (v/v) paraformaldehyde for 20 min and stained for 20 min with 0.1% crystal violet. The number of migrated cells was taken by an inverted microscope. For the cell invasion assay, chambers were precoated with Matrigel (BD Biosciences).
2.9 RNA immunoprecipitation (RIP) and methylated RNA immunoprecipitation (MeRIP)

RIP assays were conducted using an EZ Magna RIP kit (Millipore) with the Protein A/G Agarose Beads (Santa Cruz) according to the manufacturer’s instructions. The cells were lysed, and the lysates were incubated with magnetic beads conjugated with the HNRNPA2B1-specific antibody or control IgG antibody for 36 h at 4°C. The beads were incubated with proteinase K after washing with wash buffer, the purified RNA was eluted and analyzed for the presence of LINC01833 4 by qRT-PCR.

2.10 RNA m6A quantification

The m6A content in the total RNAs was measured using an m6A RNA methylation quantification kit (ab185912; Abcam). Two hundred ng RNAs were added into the assay wells. By detecting the absorbance at wavelength of 450 nm, the m6A content was quantified following the manufacturer’s protocol.

2.11 RNA pull-down assays

LINC01833 or its antisense RNA was transcribed in vitro using T7 RNA polymerase and purified using RNA PURE Kit. The transcribed LINC01833 RNA was biotinylated with a Biotin RNA Labeling Mix. Biotinylated RNAs were incubated with streptavidin-conjugated magnetic beads at room temperature. Cell was lysed, the beads were then washed, and the eluted proteins were examined by mass spectrometry and Western blot analysis.

2.12 In vivo tumor bearing mice model experiments

Male BALB/c nude mice (4 weeks old) were purchased from Beijing Vital River Company and fed in a specified pathogen-free environment. For the tumorigenicity studies, 3 × 10^6 HCC827 cells stably expressing sh-LINC01833 or sh-NC were injected subcutaneously into the ventral side of male BALB/c nude mice in the according groups with five mice in each group. Five mice were sampled each time. Tumor size and weight were examined at 28 days after injection. The volume was calculated as follows: V (mm^3) = 0.5 × D × d^2 (V is volume; D is longest diameter, and d is diameter perpendicular to the longest diameter). The tumors were excised, weighed, and collected for IHC staining. The test results were repeated three times. All protocols were approved by the Committee on the Ethics of Animal Experiments of the Shanxi Provincial Cancer Hospital and performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

2.13 Immunohistochemistry (IHC)

The tumor tissues were formalin-fixed, paraffin-embedded and cut into 3-μm sections. Next, the sections were deparaffinated with xylene and rehydration, and 10 mM citrate buffer (pH 6.0) was used for antigen retrieval for 20 min. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide. Next, samples were incubated with primary antibodies against METTL3 and HNRNPA2B1 overnight at 4°C and the biotinylated goat anti-mouse or goat anti-rabbit IgG secondary antibody (Dako) for 30 min. Next, the sections were incubated with streptavidin peroxidase for 30 min. The slides were stained with diaminobenzidine (DAB) and counterstained with hematoxylin, dehydrated by a graded ethanol series, and photographed.

2.14 Statistical analysis

The statistical analysis was performed using SPSS 21.0 software and data were presented as a mean ± standard deviation (SD). There are 3 technical repeats for all assays. An equivalent non-parametric test, the Kruskal–Wallis Test, was performed to test the assumption of normality. Homogeneity of variance was tested using Bartlett’s test. Comparisons between two or more groups were detected using Student’s t-test or one-way ANOVA followed by Tukey’s post-hoc test. A P value of less than 0.05 was considered to be statistically significant.
3. Results

3.1 LINC01833 shows upregulation in NSCLC and promotes NSCLC progression

The expression profile of LINC01833 and its functions in NSCLC were investigated. The results showed that LINC01833 expression was elevated in 30 NSCLC tissues (Figure 1a). Additionally, LINC01833 presented upregulation in NSCLC cell lines compared with the control cells. LINC01833 showed the most upregulation in the HCC827 cells (Figure 1b). Therefore, HCC827 cells was used for further studies. Subsequently, LINC01833 expression was overexpressed or suppressed in the HCC827 cells by transfection with over- or sh-LINC01833 plasmid. LINC01833 was successfully overexpressed or suppressed after transfection (Figure 1c). HCC827 cell proliferation was obviously increased in over-LINC01833 group and reduced in sh-LINC01833 group (Figure 1d). Transwell assay results revealed that migratory and invasive abilities were promoted in over-LINC01833 group and inhibited in sh-LINC01833 group (Figure 1e, f). On contrast, the apoptosis rates were significantly decreased in over-LINC01833 group while increased in sh-LINC01833 group compared with NC group (Figure 1g). This result was also confirmed by TUNEL assay (Figure 1h). These results suggested that LINC01833 promoted the NSCLC progression.

3.2 LINC01833 is modulated by m6A RNA methylation

In order to know whether LINC01833 is modulated by m6A RNA methylation, the potential m6A modification site was predicted using m6aVar online tool. There was three potential m6A modification sites on LINC01833 sequence (Figure 2a), suggesting that m6A modification might exert important effects on regulating LINC01833 during NSCLC progression. METTL3, methyltransferase 14, N6-adenosine-methyltransferase complex catalytic subunit (METTL14) and YTH N6-methyladenosine RNA

![Figure 1. LINC01833 shows upregulation in NSCLC tissue samples or cells and promotes NSCLC progression.](image-url)

(a-b) qRT-PCR was performed to detect LINC01833 expression level in the NSCLC tissue samples (n = 30) and NSCLC cells; (c) LINC01833 was overexpressed or suppressed in the HCC827 cells by transfection with over- or sh-LINC01833 plasmid; (d) CCK-8 was used to detect HCC827 cell proliferation under the transfection of over-LINC01833 and sh-LINC01833; (e-f) Transwell assays were used to detect cell migration and invasion in over-LINC01833 group and sh-LINC01833 group; (g-h) Flow cytometry and TUNEL were performed to detect cell apoptosis. Data were expressed as a mean ± SD. There are 3 technical repeats. *, P < 0.05, **, P < 0.01.
binding protein 1/2 (YTHDF1/2) are m6A modifiers in human NSCLC and play oncogenic roles in this cancer [35]. Results of RNA pulldown assays indicated that LINC01833 could interact with m6A modifier METTL3 while could not interact with METTL14 and YTHDF1/2 (Figure 2b). Results of m6A-specific immunoprecipitation assays indicated that the m6A levels of LINC01833 were increased in HCC827 cells than that in BEAS-2B cells (Figure 2c). m6A levels were reduced by sh-METTL3 and increased by over-METTL3 in HCC827 cells (Figure 2d). LINC01833 showed significantly higher level under METTL3 overexpression and lower level under METTL3 depletion in HCC827 cells (Figure 2e). Additionally, the m6A content (%) in total RNAs was higher in HCC827 than BEAS-2B (Figure 2f). These findings indicated that LINC01833 is modulated by METTL3-mediated m6A RNA methylation.

3.3 METTL3 shows elevation in NSCLC and promotes NSCLC progression

The expression profile of LINC01833 and its functions in NSCLC were subsequently explored. METTL3 level in NSCLC tissues was detected with IHC as well as Western blot. METTL3 presented upregulation in NSCLC tissues and cell lines (Figures 3A and 3b). METTL3 was successfully overexpressed or suppressed after transfection with over-METTL3 or sh-METTL3 (Figure 3c). CCK-8 assay results illustrated that HCC827 cell proliferation was obviously increased in over-METTL3 group while reduced in sh-METTL3 group (Figure 3d). Transwell assay results showed that METTL3 elevation promoted HCC827 cell migratory and invasive abilities while METTL3 depletion inhibited migration and invasion compared with NC group (Figures 3E and 3f).
On contrast, the apoptosis rates were significantly decreased in over-METTL3 group while increased in sh-METTL3 group compared with NC group (Figure 3g). This result was also confirmed by Figure 4.
TUNEL assay (Figure 3h). These results suggested that METTL3 promoted the NSCLC progression.

3.4 LINC01833 binds with HNRNPA2B1 in NSCLC cells

To clarify the regulatory mechanism of LINC01833, the RNA binding proteins of LINC01833 were examined by Starbase. The results indicated that HNRNPA2B1 is a potential RBP of LINC01833 (Figure 4a). Subsequently, the direct binding function between LINC01833 and HNRNPA2B1 protein was confirmed by RNA pull-down assays. HNRNPA2B1 was pulled down by biotinylated LINC01833 rather than negative control or anti-sense LINC01833 (Figure 4b). RIP assay results demonstrated that LINC01833 presented elevation in HNRNPA2B1 group. After knockdown of LINC01833, the binding of LINC01833 and HNRNPA2B1 protein was weakened (Figure 4c). In addition, we found that the expression of HNRNPA2B1 was reduced when knocking down LINC01833 (Figure 4d).

3.5 Inhibition of LINC01833 alleviated tumorigenesis

Role of LINC01833 was further investigated by knocking down LINC01833 in tumor xenograft mice model. The expression of LINC01833 was reduced in sh-LINC01833 group (Figure 5a). The tumor size and weight were reduced under LINC01833 knockdown (Figures 5b and 5c). Additionally, Western blot and IHC were conducted to measure METTL3 and HNRNPA2B1 protein levels. METTL3 and HNRNPA2B1 expression was reduced in tumor xenografts with LINC01833 suppression (Figures 5d and 5e). These findings suggested that knockdown of LINC01833 suppresses NSCLC progression in vivo.

4. Discussion

Multiple reports have indicated that IncRNAs exert crucial impacts on various kinds of cancer progression by regulating different cellular processes, including cycle, migration, apoptosis, and physiological changes [36]. In the previous studies, Yu et al. identified five IncRNAs which showed important

![Figure 5. Inhibition of LINC01833 alleviated tumorigenesis.](image)

(a) LINC01833 expression was reduced in sh-LINC01833 group compared with NC group; (b-c) The tumor size and weight were reduced under LINC01833 knockdown; (d-e) The expression levels of METTL3 and HNRNPA2B1 were detected by Western blot and IHC in tumor tissues. Data were presented as a mean ± SD. Each group has 5 mice. There are 3 technical repeats. **, P < 0.01.
prognostic values in NSCLC. LINC01833 showed the most significant upregulation in the NSCLC tissues compared with control group [17]. The present study shows that overexpression of LINC01833 promoted cell proliferative, invasive, and migratory capabilities, inhibited cell apoptosis, and promoted tumorigenesis, suggesting that LINC01833 can promote NSCLC progression.

m6A modification at the post-transcriptional level in the eukaryotic genome exerts an important role in the pathophysiological process of multiple cancers [18,19]. Li et al. indicated that patients with FTO and YTHDC2 deficiency have unfavorable disease-free survival and overall survival, suggesting the prognostic value of these m6A RNA methylation regulators in NSCLC [37]. Yin et al. showed that m6A methylation controls RMRP stability to render NSCLC progression [22]. Recently, Lei et al. showed that ABHD11-AS1 showed upregulation in NSCLC tissue samples and cells. METTL3 stabilizes ABHD11-AS1 transcript to upregulate ABHD11-AS1 by installing the m6A modification [24]. In the present study, we found that LINC01833 is modulated by METTL3-mediated m6A RNA methylation, suggesting that the METTL3-mediated m6A RNA methylation plays crucial roles in the regulation of LINC01833 on NSCLC progression.

HNRNPA2B1 is an important RNA binding protein affecting the RNA splicing and transport and stability of its downstream target genes. HNRNPA2B1 exerts crucial impacts on lung cancers. Dai et al. [32] and Dowling et al. [38] demonstrated that abnormal levels of HNRNPA2B1 could act as an important diagnose indicator for lung cancer patients. Yu et al. demonstrated that CACNA1G-AS1 promoted NSCLC cell migratory and invasive abilities through regulating HNRNPA2B1 [39]. Recently, Jin et al. indicated that HNRNPA2B1 promoted NSCLC malignancy through facilitating cell growth and metastasis, suggesting that HNRNPA2B1 is a promising targeted biomarker for therapy of NSCLC [40]. Herein, LINC01833 could bind to HNRNPA2B1 in NSCLC cells. In consistent with previous reports, HNRNPA2B1 overexpression promoted NSCLC cell proliferative, invasive, and migratory capabilities and inhibited apoptosis. In addition, HNRNPA2B1 is recognized as a modulator of m6A-dependent RNA processing events in nucleus. HNRNPA2B1 directly interacts with a set of nuclear transcripts and elicits similar alternative splicing function as the m6A writer METTL3 [41]. Therefore, we concluded that METTL3-induced LINC01833 promoted the NSCLC progression by interacting with HNRNPA2B.

Interestingly, it was found that the upstream modulator of LINC01833, METTL3, is suppressed by LINC01833 knockdown in in vivo studies. HNRNPA2B1 is an RNA binding protein and may bind with mRNAs to stabilize or destabilize their expression. We used the starbase tool to identify the downstream targets of HNRNPA2B1 and found 11,130 mRNAs. Proteins of 100 mRNAs among these 11,130 mRNAs were predicted as transcription factors for METTL3 (Supplementary material 4). LINC01833 suppression-induced reduction of METTL3 may be associated with the 100 genes that were regulated by HNRNPA2B1 (positively regulated by LINC01833) and spontaneously modulated METTL3.

Conclusion

m6A transferase METTL3-triggered LINC01833 m6A methylation facilitates NSCLC progression through modulating HNRNPA2B1, which provides a novel direction for the better understanding of the pathogenesis of NSCLC.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The author(s) reported there is no funding associated with the work featured in this article.

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