METTL3-Mediated m\textsuperscript{6}A Modification of lncRNA MALAT1 Facilitates Prostate Cancer Growth by Activation of PI3K/AKT Signaling

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Abstract

Accumulating data show that N6-methyladenosine (m\textsuperscript{6}A) methyltransferase METTL3 and long noncoding RNA MALAT1 act pivotal roles in multiple malignancies including prostate cancer (PCa). However, the role and molecular mechanism underlying METTL3-mediated m\textsuperscript{6}A modification of MALAT1 in PCa remain undocumented. The association of METTL3 and MALAT1 expression with clinicopathological characteristics and prognosis in patients with PCa was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR), Western blot, and public The Cancer Genome Atlas (TCGA) dataset. The in vitro and in vivo experiments were executed to investigate the role of METTL3 in PCa. m\textsuperscript{6}A dot blot, methylated RNA immunoprecipitation (MeRIP), RIP, and qRT-PCR assays were employed to observe METTL3-mediated m\textsuperscript{6}A modification of MALAT1. The effects of METTL3 on MALAT1-mediated PI3K/AKT pathway were assessed by Western blot analysis. As a result, we found that METTL3 was significantly upregulated in PCa tissues and high expression of METTL3 was associated with Gleason score and tumor recurrence in patients with PCa. Knockdown of METTL3 markedly repressed growth and invasion of PCa cells in vitro and in vivo, whereas ectopic expression of METTL3 showed the opposite effects. Moreover, knockdown of METTL3 decreased the total m\textsuperscript{6}A levels of PCa cells as well as the MALAT1 m\textsuperscript{6}A levels, leading to reduced MALAT1 expression. Overexpression of MALAT1 reversed METTL3 knockdown-induced antitumor effects and PI3K/AKT signaling inactivation. MALAT1 harbored a positive correlation with METTL3 expression and tumor recurrence in PCa. In conclusion, our findings demonstrate that METTL3-mediated m\textsuperscript{6}A modification of IncRNA MALAT1 promotes growth and invasion of PCa cells by activating PI3K/AKT signaling.

Keywords
m\textsuperscript{6}A, METTL3, MALAT1, prostate cancer, growth

Introduction

Prostate cancer (PCa) is the most common malignancy in men and constructs the second leading cause of cancer-related death in men worldwide\textsuperscript{1}. Despite the great advances made in cancer therapy, advanced patients with PCa still possess the poor prognosis ascribed to the tumor invasiveness and metastasis\textsuperscript{2}. Epigenetic regulations including RNA modifications and dysregulation of noncoding RNA are implicated in gene expression and cancer progression\textsuperscript{3}. Therefore, comprehensive observations of the molecular mechanisms underlying PCa progression are essential for the effective therapeutic strategies.

N6-methyladenosine (m\textsuperscript{6}A), one of the most abundant mRNA modifications, is catalyzed by the methyltransferase complex including methyltransferase-like 3/14 (METTL3/14),
and dynamically reversed by demethylase fat mass and obesity associated (FTO) and ALKBH5. Increasing data uncover that METTL3 acts a critical role in cancer progression and treatment. METTL3 facilitates cancer growth and metastasis by promoting m6A-dependent pri-miR221/222/1246 maturation, mediates m6A modification of HDGF/SPHK2/SOX2 contributing to cancer progression and poor prognosis, and regulates oncogene translation and immune responses in cancer. STM2457, a potent and selective inhibitor of METTL3, provides a novel strategy against acute myeloid leukemia. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) as a long noncoding RNA (lncRNA) acts a multifunctional role in cancer. It has been reported that MALAT1 promotes cancer cell growth, invasion, and metastasis and regulates chemoresistance by sponging miR-3064-5p/-613/-204/-27a-5p, binding to splicing factor proline and glutamine rich (SFPQ) and impairing β-catenin degradation. MALAT-1 facilitates PCa progression and represents a potential therapeutic target. Targeting MALAT1 by nanocomplex carrying siRNA sensitizes glioblastoma to temozolomide. Recent studies reveal that METTL3/SNHG1/miR-140-3p axis exerts prognostic and immunological roles in non-small-cell lung cancer (NSCLC) and METTL3-mediated lncRNA FOXD2-AS1 and ABHD11-AS1 aggravate the tumorigenesis of cervical cancer and the Warburg effect of NSCLC. However, the role and molecular mechanism underlying METTL3-mediated MALAT1 in PCa remain undocumented. We herein found that METTL3-mediated m6A modification of lncRNA MALAT1 promoted proliferation and invasion of PCa cells by activating PI3K/AKT signaling, and might provide a therapeutic target for PCa.

**Methods**

**Clinical Samples**

In all, 484 cases of non-paired PCa tissue samples and 52 pair-matched samples were collected from The Cancer Genome Atlas (TCGA) database (http://xena.ucsc.edu/getting-started/). Ten pairs of PCa samples, used for polymerase chain reaction (PCR) and Western blot assays were preserved in liquid nitrogen and frozen at −80°C. Our study protocol was approved by the Ethics Committee of Shanghai Ninth People’s Hospital.

**RNA Extraction and Real-Time Quantitative PCR**

Total RNA was collected by using a RNA extraction kit (QIAGEN, Dusseldorf, Germany), and cDNA was synthesized by using a reverse transcription kit (Promega, Madison, USA) according to the manufacturer’s instructions. PCR assay was performed using the SYBR Green Master Mix. After the reactions were finished, the relative expression levels of METTL3 and MALAT1 were calculated using the 2^−ΔΔCt. The primer sequences used were shown in Supplementary Table S1.

**Western Blot**

PCa tissue samples and DU145 and 22RV1 cells were lysed by RIPA (radio-immunoprecipitation assay) buffer (P0013B; Beyotime, Shanghai, China). The supernatants were resolved in SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (IPVH0010; Millipore, Billerica, MA, USA), incubated with anti-METTL3 (DF12020; Affinity, Changzhou, China), anti-PI3K (AF6241, Affinity), anti-AKT (60203-2-Ig; Proteintech, Wuhan, China), anti-p-AKT (66444-1-Ig; Proteintech), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AB-P-R 001; Hangzhou, China) overnight at 4°C. Protein bands were scanned by enhanced chemiluminescence (ECL).

**Plasmid, shRNA, and Cell Transfection**

METTL3 plasmid vector, lentivirus-mediated shRNA targeting METTL3 (sh-METTL3, 5′-GCAAGAATTCTGTGACTATGG-3′), and MALAT1 plasmids were purchased from GenePharma (Shanghai, China). The negative vector (Vector) and sh-NC were regarded as the control groups. DU145 and 22RV1 cells were planted in six-well plates 24 h prior to sh-METTL3, METTL3 plasmids, and MALAT1 plasmids transfection with 50% to 60% confluence, and then mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacture instructions.

**Cell Culture, MTT, EdU, Transwell Assays, and Immunohistochemistry Analysis**

These assays were performed as previously reported.

**RNA Immunoprecipitation**

Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (Millipore) was used. DU145 and 22RV1 cells were lysed in RNA immunoprecipitation (RIP) lysis buffer, and then cell extracts were incubated with magnetic beads labeled with the antibodies of Argonaute2 (Ago2; Millipore), METTL3 (Affinity), and Immunoglobulin G (IgG; Millipore). RNAs that were pulled-down were extracted using Trizol reagent (Invitrogen). RNA expression was determined by real-time quantitative PCR (RT-qPCR).

**Methylated RNA Immunoprecipitation Assay**

Methylated RNA immunoprecipitation (MeRIP) assay was performed according to the MeRIP Kit (BersinBio, Guangzhou, China) was used. Briefly, RNA was broken into about 300 bp fragments, and 50 μl was separated as input. The remaining samples were divided into two groups...
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and incubated with m^6^A (A-1801-020; Epigentek, Wuhan, China) and IgG antibodies, respectively. The enriched RNA was extracted after immunoprecipitation, and qPCR was used for subsequent experimental analysis after RNA reverse transcription.

**In Vivo Tumorigenesis Assay**

The female Balb/C-nu/nu nude mice (6–8 weeks old, 18–20 g) were purchased from the Shanghai Laboratory Animal Central. DU145 cells (2 × 106) transfected with the stable transfection of sh-METTL3 lentivirus or sh-NC were resuspended in 200 μl of sterile PBS and injected subcutaneously into the right flanks of mice. After 5 weeks, the mice were killed, and the tumor volume and weight were calculated. The animal experiments were approved by the Ethics Committee of Shanghai Ninth People’s Hospital.

**Statistical Analysis**

Statistical analyses were processed with GraphPad Prism 7 (La Jolla, CA, USA). The values are expressed as the mean ± standard deviation. Chi-square, Student’s t test, and analysis of variance were used for comparisons between groups. Kaplan–Meier analysis was used to analyze the correlation of METTL3 or MALAT1 with PCa. A Cox proportional hazard model was used to estimate the risk of METTL3 or MALAT1 in PCa. Pearson correlation analysis was used to analyze the correlation of METTL3 and MALAT1. P < 0.05 was considered statistically significant.

**Results**

**The Upregulation of METTL3 Indicates Tumor Recurrence in Patients With PCa**

The RNA expression levels of METTL3 in PCa tissue samples were assessed by TCGA cohort, which indicated that METTL3 was upregulated in both 52 pare-matched PCa tissue samples and 484 non-pairs of PCa samples as compared with adjacent normal tissues (Fig. 1A). The upregulation of METTL3 in PCa was further validated in our cohort by qRT-PCR (Fig. 1B) and Western blot assays (Fig. 1C). Then, according to the survival time, survival status, and METTL3 expression levels, we acquired the cutoff value of METTL3 (9.723) in PCa patients from TCGA cohort by a Cutoff Finder and divided the patients into METTL3-high group (n = 278) and METTL3-low group (n = 116). It was shown that elevation of METTL3 expression was associated with Gleason score (P < 0.001) in PCa patients from TCGA cohort (Supplementary Table S2). Kaplan–Meier analysis revealed that the PCa patients with METTL3-high expression harbored higher tumor recurrence as compared with those with METTL3-low expression (Fig. 1D), but had no difference in overall survival (Fig. S1). Subsequently,
multivariate Cox regression analysis unveiled that METTL3 high expression was not an independent risk factor for tumor recurrence in patients with PCa from TCGA cohort (Supplementary Table S3).

**Knockdown of METTL3 Dampens PCa Cell Growth and Invasion**

The transfection levels of lentivirus-mediated sh-METTL3 were determined by qRT-PCR and Western blot assays, which indicated that the METTL3 expression levels were markedly decreased in DU145 and 22RV1 cell lines after transfection with sh-METTL3 for 48 h (Fig. 2A). Cell proliferation and DNA synthesis were assessed by MTT and EdU assays, which indicated that knockdown of METTL3 repressed cell proliferation activity (Fig. 2B) and DNA synthesis (Fig. 2C) in DU145 and 22RV1 cells. Transwell was used to evaluate cell invasive capabilities, indicating that inhibition of METTL3 prohibited cell invasive potential in DU145 and 22RV1 cells (Fig. 2D).

**Overexpression of METTL3 Promotes PCa Cell Growth and Invasion**

The transfection levels of METTL3 plasmids were defined by qRT-PCR and Western blot assays, which showed that the METTL3 expression levels were dramatically increased in DU145 and 22RV1 cells after transfection with METTL3 plasmids for 48 h (Fig. 3A). Cell proliferation and DNA synthesis were estimated by MTT and EdU assays, which
indicated that METTL3 overexpression enhanced cell proliferation activity (Fig. 3B) and DNA synthesis (Fig. 3C) in DU145 and 22RV1 cells. Transwell was used to evaluate cell invasive capabilities, implying that overexpression of METTL3 drove cell invasive potential in DU145 and 22RV1 cells (Fig. 3D).

**LncRNA MALAT1 is an m^6^A Target of METTL3 in PCa Cells**

We identified the binding RNAs of METTL3 by using starbase3.0 (https://starbase.sysu.edu.cn) and found that METTL3 had the most binding sites with LncRNA MALAT1. We proposed that METTL3 might mediate the m^6^A modification of MALAT1. The m^6^A dot blot showed that knockdown of METTL3 reduced the whole m^6^A levels in DU145 and 22RV1 cells (Fig. 4A). MeRIP and qRT-PCR further verified that knockdown of METTL3 decreased the m^6^A levels of MALAT1 (Fig. 4B) as well as its mRNA levels (Fig. 4C) in DU145 and 22RV1 cells. Furthermore, we conducted RIP assay for METTL3-specific binding with MALAT1 in DU145 and 22RV1 cells and investigated the endogenous levels of MALAT1 pulled-down from METTL3-expressed cells by qRT-PCR analysis, which indicated that MALAT1 was obviously enriched in the METTL3 pellet compared with those in the IgG control (Fig. 4D).
The Upregulation of MALAT1 Indicates Tumor Recurrence in Patients With PCa

The expression of MALAT1 in PCa tissue samples was estimated by TCGA cohort, which indicated that MALAT1 expression was significantly increased in both 51 paired PCa tissue samples and 481 non-pairs of PCa samples as compared with adjacent normal tissues (Fig. 5A). The expression of MALAT1 was elevated in PCa with Gleason (≥8) relative to those with Gleason (= 7) (Fig. 5B). The upregulation of MALAT1 was further validated in 10 pairs of PCa tissue samples by qRT-PCR analysis (Fig. 5C). Pearson correlation analysis showed that METTL3 had a positive correlation with MALAT1 expression in PCa (Fig. 5D). We obtained the cutoff value of MALAT1 (11.96) in PCa patients and divided the patients from TCGA cohort into MALAT1-high group (n = 169) and MALAT1-low group (n = 122). Then, high expression of MALAT1 was associated with Gleason score (P = 0.007) and lymph node metastasis (P = 0.017) in PCa patients from TCGA cohort (Supplementary Table S5). Kaplan–Meier analysis revealed that the PCa patients with MALAT1-high expression possessed higher tumor recurrence as compared with those with MALAT1-low expression (Fig. 5E), but had no difference in overall survival (Fig. S2). Multivariate Cox regression analysis unveiled that MALAT1 high expression was not an independent risk factor for overall survival and tumor recurrence in patients with PCa from TCGA cohort (Supplementary Tables S5 and S6).

MALAT1 Abrogates METTL3 Knockdown-Induce Antitumor Effect and PI3K/AKT Inactivation

To further assess the regulation of METTL3 on MALAT1 in PCa cells, we constructed MALAT1-overexpressed DU145 and 22RV1 cells, indicated by qRT-PCR analysis (Fig. 6A). We co-transfected MALAT1 plasmids and sh-METTL3 lentiviruses into DU145 and 22RV1 cells and found that MALAT1 overexpression could reverse METTL3 knockdown-induced inhibitory effects on cell proliferation, invasion and DNA synthesis in DU145 and 22RV1 cells (Fig. 6B–D). Moreover, Western blot analysis displayed that knockdown of METTL3 could markedly repress the phosphorylated PI3K/AKT activities rather than the total PI3K/AKT expression, and overexpression of MALAT1 could reverse METTL3 knockdown-caused PI3K/AKT inactivation in DU145 and 22RV1 cells (Fig. 6E).
Figure 5. Upregulation of MALAT1 is associated with tumor recurrence in patients with PCa. (A) TCGA analysis of the expression levels of MALAT1 in 51 pair-matched and 481 non-paired PCa tissue samples. (B) TCGA analysis of the expression levels of MALAT1 in patients with diverse Gleason scorings. (C) qRT-PCR analysis of the expression levels of MALAT1 in 10 pairs of PCa tissue samples. (D) Pearson correlation analysis of the correlation of METTL3 with MALAT1 in PCa tissue samples. (E) TCGA cohort analysis of the association of MALAT1 with the prognosis in PCa with patients. PCa: prostate cancer; qRT-PCR: quantitative real-time polymerase chain reaction.

Figure 6. Overexpression of MALAT1 abolishes METTL3 knockdown-induced antitumor effect and PI2K/AKT signaling inactivation. (A) qRT-PCR analysis of the transfection efficiency of MALAT1 plasmids in DU145 and 22RV1 cells. (B) MTT analysis of the cell proliferation activities after co-transfection with MALAT1 plasmids and sh-METTL3 into DU145 and 22RV1 cells. (C) Transwell analysis of the cell invasion capabilities after co-transfection with MALAT1 plasmids and sh-METTL3 into DU145 and 22RV1 cells. (D) EdU analysis of the DNA synthesis after co-transfection with MALAT1 plasmids and sh-METTL3 into DU145 and 22RV1 cells. (E) Western blot analysis of the PI3K/AKT signaling activities after co-transfection with MALAT1 plasmids and sh-METTL3 into DU145 and 22RV1 cells. Data are the means ± SEM of three experiments. qRT-PCR: quantitative real-time polymerase chain reaction. *P < 0.05; ***P < 0.001; ****P < 0.0001.
Knockdown of METTL3 Suppresses In Vivo Tumorigenesis

To decipher whether METTL3 affects in vivo tumor growth, we constructed a sh-METTL3 or sh-NC stably transfected DU145 cells, and then subcutaneously injected into the flank of nude mice. After investigations for 5 weeks, it was found that the tumor volumes of sh-METTL3-transfected group were smaller than the sh-NC group (Fig. 7A). The curve for tumor growth implied that the tumors in sh-METTL3 transfected group showed a decrease in a time-dependent manner (Fig. 7B), and the volume and weight of xenograft tumors were markedly weakened in sh-METTL3 transfected group as compared with the sh-NC group (Fig. 7C, D). Further analyses of qRT-PCR and immunohistochemistry (IHC) showed that knockdown of METTL3 reduced the expression of MALAT1 (Fig. 7E) and inhibited the activation of p-PI3K and p-AKT (Fig. 7F, G) in xenograft tumor tissues.

Discussion

Accumulating studies indicate that m6A methylation modification is essential to maintain cancer growth and metastasis\(^8,^{25}\). METTL3 is upregulated in bladder cancer\(^6\), cervical cancer\(^{26}\), colorectal cancer (CRC)\(^{27}\), oral squamous cell carcinoma\(^{28}\), and associated with lymph node metastasis and poor prognosis\(^{6,26–28}\). METTL3 expression is also elevated in metastatic PCa and correlated with malignant progression and poor prognosis in PCa\(^{29}\). However, METTL3 is downregulated in papillary thyroid cancer (PTC), restrains tumor growth, and indicates favorable prognosis in patients with PTC\(^{30}\). In the present study, we found that METTL3 was upregulated in PCa and associated with Gleason score and tumor recurrence in patients with PCa, suggesting that METTL3 may be a potential prognostic factor in PCa.

Previous studies showed that, on one hand, METTL3 acts as an oncogene in multiple malignancies\(^{6–10,26–28}\).
On the other hand, METTL3 functions as a tumor suppressor in renal cell carcinoma\textsuperscript{33} and enhances cisplatin chemosensitivity of cervical cancer by downregulation of RAGE\textsuperscript{32}. In PCa tissues, METTL3-mediated m\textsuperscript{6}A modification of KIF3C and MYC facilitates PCa progression\textsuperscript{33,34} and inhibition of METTL3 impairs invasion and metastasis of PCa\textsuperscript{35}. In consistence with these studies\textsuperscript{33–35}, we herein found that knockdown of METTL3 suppressed PCa cell proliferation and invasion in vitro and in vivo, whereas overexpression of METTL3 had the opposite effects, indicating that METTL3 may be an oncogenic factor in PCa.

Mechanical investigations unveil that METTL3 can mediate m\textsuperscript{6}A modification of noncoding RNAs including miRNA\textsuperscript{6,7} and lncRNA\textsuperscript{22–24} involved in cancer progression. MALAT1 has been verified as a crucial tumorigenic factor in PCa\textsuperscript{14–20}, and METTL3-mediated m\textsuperscript{6}A modification of MALAT1 promotes growth and metastasis in NSCLC\textsuperscript{36} and glioma\textsuperscript{37}. We herein found that knockdown of METTL3 reduced the m\textsuperscript{6}A modification level of MALAT1, which reversed METTL3 knockdown-induced antitumor effects and indicated tumor recurrence in PCa. These findings suggested that METTL3-mediated m\textsuperscript{6}A modification of MALAT1 promoted PCa growth.

In addition, MALAT1 facilitates cell proliferation, invasion, and cisplatin resistance by activating PI3K/AKT pathway in gastric cancer and cholangiocarcinoma\textsuperscript{38–40}. MALAT1/miR-146a/PI3K/AKT pathway repress cell apoptosis and autophagy in hepatocellular carcinoma (HCC)\textsuperscript{41} and MALAT1/miR-26a/26b/FUT4/PI3K/AKT pathway promotes CRC invasion and metastasis\textsuperscript{42}. METTL3 promotes retinoblastoma growth and endothelial progenitor cell angiogenesis by PI3K/AKT pathway\textsuperscript{43,44}. We herein found that MALAT1 promoted the activation of PI3K/AKT signaling and abrogated METTL3 knockdown-induced PI3K/AKT signaling inactivation in PCa cells.

In conclusion, our findings demonstrate that METTL3 upregulation is associated with Gleason scoring and tumor recurrence in patients with PCa and METTL3-mediated m\textsuperscript{6}A modification of lncRNA MALAT1 promotes PCa tumorigenesis by activation of PI3K/AKT signaling. Our present study may provide potential therapeutic strategies for treatment of PCa.

**Ethical Approval**
This study was approved by the Ethics Committee of Shanghai Ninth People’s Hospital.

**Statement of Human and Animal Rights**
All procedures in this study were conducted in accordance with the Ethics Committee of Shanghai Ninth People’s Hospital.

**Statement of Informed Consent**
Written informed consent was obtained from the patients for their anonymized information to be published in this article.

**Declaration of Conflicting Interests**
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the National Natural Science Foundation of China (81970656) and Seed Funding of Shanghai Ninth People’s Hospital, Shanghai Jiao Tong university School of Medicine (JYZZ113).

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**Supplemental Material**
Supplemental material for this article is available online.

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