

ORIGINAL ARTICLE

The m⁶A methyltransferase METTL3 promotes the stemness and malignant progression of breast cancer by mediating m⁶A modification on SOX2

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Summary

Purpose: We aimed to uncover the role of METTL3 in stimulating the stemness and progression of breast cancer (BCa) through mediating N⁶-methyladenosine (m⁶A) modification on SOX2 mRNA.

Methods: METTL3 levels in 48 paired BCa and adjacent normal ones were examined. Kaplan-Meier method was introduced for assessing the prognostic value of METTL3 in BCa. Regulatory effects of METTL3 on invasive and migratory abilities in MCF-7 cells were evaluated by Transwell assay. Besides, the protein levels of SOX2 and tumor stem cell markers CD133 and CD44 in MCF-7 cells affected by METTL3 were determined by Western blot. In addition, the potential interaction between METTL3 and SOX2 was ascertained through RIP (RNA-Binding Protein Immunoprecipitation) assay. Moreover, the interaction between IGF2BP2 and SOX2 influenced by METTL3 was verified by RIP assay as well.

Results: METTL3 was upregulated in BCa tissues, especial-

ly in T3-T4 or those accompanied with lymphatic metastasis. BCa patients expressing a high level of METTL3 suffered worse prognosis. Knockdown of METTL3 downregulated protein levels of SOX2, CD133 and CD44 in MCF-7 cells. Moreover, invasive and migratory abilities were attenuated in BCa cells with METTL3 knockdown. Silencing of IGF2BP2 markedly downregulated SOX2. RIP assay confirmed the binding between METTL3 and SOX2 mRNA, and knockdown of METTL3 decreased the enrichment of SOX2 in anti-IGF2BP2. Interestingly, overexpression of SOX2 partially reversed the regulatory effects of downregulated METTL3 on MCF-7 cells.

Conclusions: METTL3 is upregulated in BCa, and it promotes the stemness and malignant progression of BCa through mediating m⁶A modification on SOX2 mRNA.

Key words: m⁶A, METTL3, SOX2, breast cancer

Introduction

Breast cancer (BCa) is a major health problem even though great strides have been made on its research [1]. BCa is a highly prevalent malignant cancer in American women only second to skin cancer, and it is also the second leading fatal malignancy following lung cancer [2]. Prevention and treatment of BCa are still critical.

The epigenetic regulation N⁶-methyladenosine (m⁶A) is currently on the medical research front [3]. As the most abundant modification, m⁶A modi-

fication requires three types of enzymes, namely, writers, erasers and readers. Writers first catalyze m⁶A methylation on targeting mRNAs, and erasers subsequently mediate the specific modification. At last, readers are responsible for recognizing the modified information, and thus participate in translation and degradation of downstream RNAs [4-6]. Previous studies have demonstrated the extensive effects of m⁶A on embryonic development, DNA damage repair, tumor progression, etc. [7-9].

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Breast cancer stem cells (BCSCs) present tumor initiation potential and self-renewal ability, which are fundamental cells influencing drug resistance, tumor metastasis and recurrence [10,11]. In recent decades, CSC-targeting strategies have presented therapeutic efficacy on BCa, some of which are currently being evaluated in clinical trials [10,12,13]. Therefore, CSCs may be utilized for developing novel therapeutic drugs for cancer treatment.

Methods

Sample collection

BCa tissues and adjacent normal ones (3 cm away from the tumor tissues) were surgically resected from 48 BCa patients undergoing treatment in our hospital. None of BCa patients received preoperative anti-tumor therapy. Tissue samples were immediately frozen in liquid nitrogen and preserved at -80°C . Patients and their families in this study have been fully informed. This study was approved by Ethics Committee of Affiliated Zhongshan Hospital of Dalian University.

Cell culture

MCF10A, MCF-7 and BT474 cells purchased from Cell Bank (Shanghai, China), were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 $\mu\text{g}/\text{mL}$ streptomycin and 100 IU/mL penicillin (Invitrogen, Carlsbad, CA, USA). They were maintained at 37°C , 5% CO_2 . Culture medium was regularly replaced.

Cell transfection

Cells were washed, digested and centrifuged. Cell suspension was applied in 6-well plates and grown to 60% confluence. Then transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and fresh medium was replaced 6 hours later.

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA, which was quantified and qualified using a spectrometer. RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the PrimeScript RT reagent Kit (TaKaRa,

Tokyo, Japan). SYBR Premix Ex Taq TM (TaKaRa, Tokyo, Japan) was utilized for qRT-PCR. Relative level was calculated using $2^{-\Delta\Delta\text{Ct}}$ method. Primer sequences were listed in Table 1.

Western blot

Cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Transwell assay

A total of 3×10^4 cells/per were inoculated on the top of a Transwell insert placed in a 24-well plate, and 100 μL of serum-free medium was applied in the bottom. After 24-h incubation, cells penetrating to the bottom were immersed in methanol for 15 min, dyed with crystal violet for 20 min and counted using a microscope. Penetrating cells were counted in 5 randomly selected fields per sample (magnification 40 \times). Thereafter, migration assay was similarly performed with 100 μL of diluted Matrigel pre-coating in the inner side of each insert and 50 μL of FN pre-coating in the bottom.

RIP (RNA-Binding Protein Immunoprecipitation) assay

Cells were collected and processed according to the procedures of Millipore Magna RIP Kit (Millipore, Billerica, MA, USA). Cells were incubated with the input, corresponding antibodies or anti-IgG at 4°C overnight. A protein-RNA complex was obtained after capturing intracellular specific proteins by the antibody. Subsequently, proteins were digested by proteinase K and the RNAs were extracted. During the experiment, the magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. The immunoprecipitant RNA was finally subjected to qRT-PCR for determining the relative level.

Statistics

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Data were expressed as mean \pm standard deviation. T-test was used for analyzing differences between two groups. Survival analysis was conducted by Kaplan-Meier method, followed by Log-rank test. $P < 0.05$ indicated the significant difference.

Results

METTL3 was upregulated in BCa and predicted poor prognosis

Compared with adjacent normal tissues, METTL3 was upregulated in BCa tissues (Figure 1A). In particular, METTL3 level was higher in T3-T4 BCa patients compared with T1-T2 patients (Figure 1B). A higher level of METTL3 was observed in

Table 1. Primer sequence

Gene	Primer sequence
SOX2	F: 5'- AATTGCACTTCGCCCCGTCT-3' R: 5'- ATCATGCTGTAGCTGCCGTT-3'
METTL3	F: 5'- AGATGGGGTAGAAAGCCTCCT-3' R: 5'- TGGTCAGCATAGGTTACAAGAGT-3'
GAPDH	F: 5'-CGGAGTCAACGGATTTGGTCGT-3' R: 5'-GGGAAGGATCTGTCTCTGACC-3'

BCa patients accompanied with lymphatic metastasis relative to those without lymphatic metastasis (Figure 1C). Besides, survival analysis revealed a poor prognosis in BCa patients expressing high level of METTL3 (HR=2.818; P=0.0258).

Knockdown of METTL3 alleviated the malignant progression of BCa

Compared with normal breast cell line MCF10A, METTL3 was upregulated in BCa cell lines MCF-7 and BT474 (Figure 2A). To elucidate the biological function of METTL3, si-METTL3 was constructed and its transfection efficacy was tested in MCF-7 cells (Figure 2B). It was discovered that protein levels of SOX2 and stem cell markers CD133 and CD44 were downregulated in MCF-7 cells transfected with si-METTL3 (Figure 2C). Tran-

swell assay further depicted the attenuated invasive and migratory abilities in MCF-7 cells after knockdown of METTL3 (Figure 2D). The above results suggested that knockdown of METTL3 suppressed stemness and metastasis in BCa.

METTL3 induced m⁶A modification on SOX2 mRNA

Interestingly, protein level of SOX2 was downregulated in MCF-7 cells transfected with si-IGF2BP2 (Figure 3A). As RIP assay depicted, SOX2 was mainly enriched in anti-METTL3 compared with that of anti-IgG, suggesting the interaction between METTL3 and SOX2 (Figure 3B). Besides, it was also demonstrated that IGF2BP2 could interact with SOX2 mRNA (Figure 3C). Knockdown of METTL3 markedly decreased the enrichment abundance of SOX2 in anti-IGF2BP2 (Figure 3D).

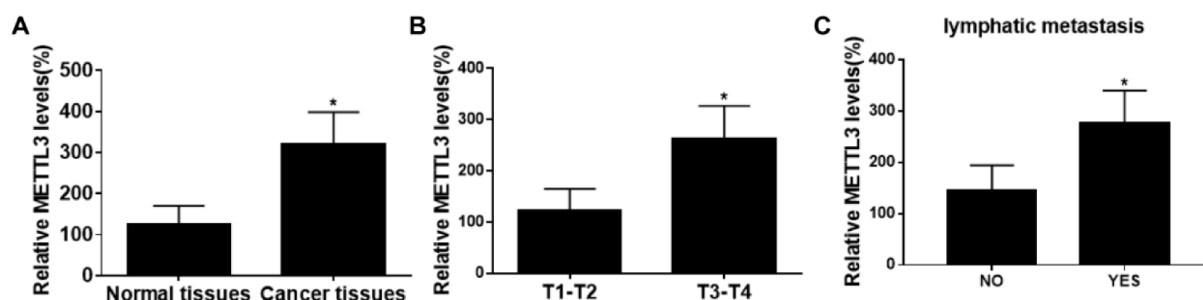


Figure 1. METTL3 was upregulated in BCa and predicted poor prognosis. **A:** METTL3 levels in normal tissues and BCa tissues. **B:** METTL3 levels in BCa patients with T1-T2 or T3-T4. **C:** METTL3 levels in BCa patients either with lymphatic metastasis or not (*p<0.05).

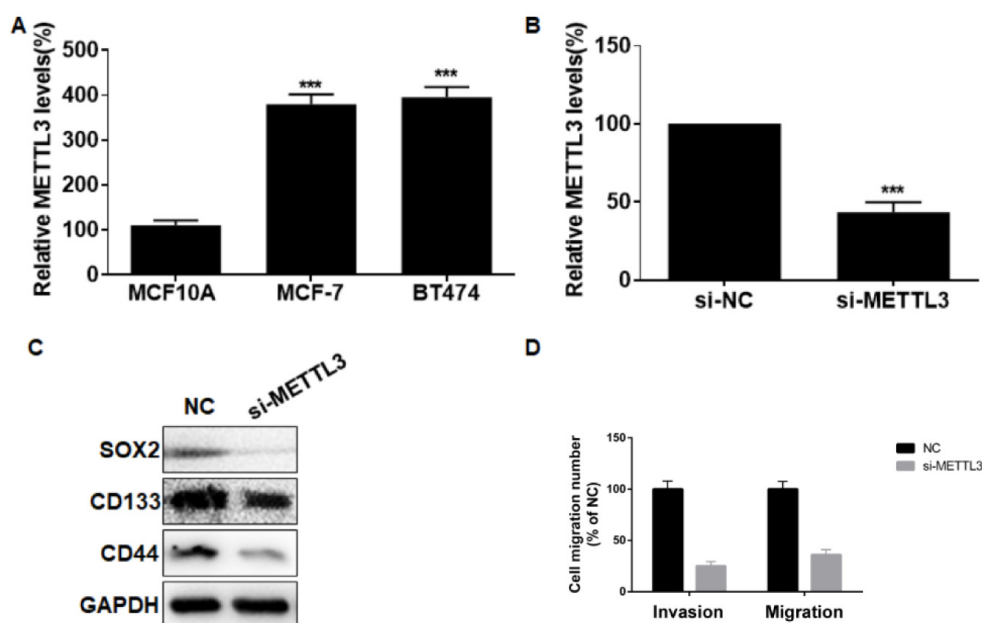


Figure 2. Knockdown of METTL3 alleviated the malignant progression of BCa. **A:** METTL3 levels in normal breast cells MCF10A and BCa cells MCF-7 and BT474 cells. **B:** Transfection efficacy of si-METTL3 in MCF-7 cells. **C:** Protein levels of SOX2, CD133 and CD44 in MCF-7 cells transfected with NC or si-METTL3. **D:** si-METTL3 remarkably inhibited the invasion and migration of MCF-7 cells (**p<0.001).

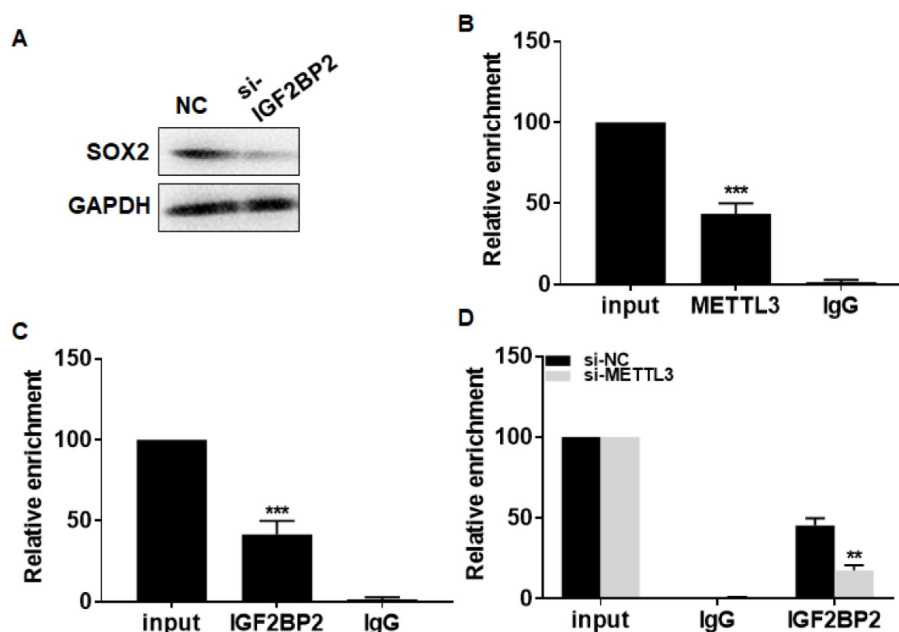


Figure 3. METTL3 induced m⁶A modification on SOX2 mRNA. **A:** Protein level of SOX2 in MCF-7 cells transfected with NC or si-IGF2BP2. **B:** Relative enrichment of SOX2 in input, anti-METTL3 and anti-IgG. **C:** Relative enrichment of SOX2 in input, anti-IGF2BP2 and anti-IgG. **D:** Relative enrichment of SOX2 in input, anti-IGF2BP2 and anti-IgG after transfection of NC or si-METTL3 in MCF-7 cells (**p<0.01, ***p<0.001).

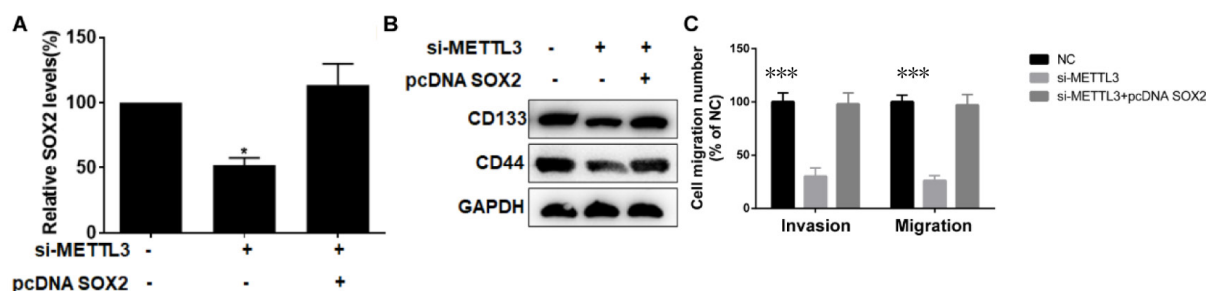


Figure 4. METTL3 regulated malignant progression of BCa through SOX2. MCF-7 cells were transfected with NC, si-METTL3 or si-METTL3 + pcDNA SOX2. **A:** Relative level of SOX2; **B:** Protein levels of CD133 and CD44; **C:** Knockdown of METTL3 attenuated invasion and migration of MCF-7 cells, while overexpression of SOX2 blocked these effects (*p<0.05, ***p<0.001).

The above data proved that METTL3 induced m⁶A modification on SOX2 mRNA, thereby regulating protein level of SOX2.

METTL3 regulated malignant progression of BCa through SOX2

Based on the above findings, we thereafter speculated that SOX2 was responsible for METTL3-regulated malignant progression of BCa. We first constructed pcDNA SOX2 and tested its overexpression efficacy (Figure 4A). The downregulated CD133 and CD44 in MCF-7 cells transfected with si-METTL3 were partially reversed after co-transfection of pcDNA SOX2 (Figure 4B). In addition, knockdown of METTL3 attenuated invasive and migratory abilities in MCF-7 cells, which were

blocked by overexpression of SOX2 (Figure 4C). The above findings confirmed that SOX2 was involved in METTL3-regulated stemness and malignant level of BCa.

Discussion

METTL3, as the key component of m⁶A methylation complex, is critical in many types of cancer [14-17]. In this paper, METTL3 was upregulated in BCa tissues and cell lines. Moreover, patients with advanced BCa accompanied with lymphatic metastasis or with poor prognosis usually expressed a high level of METTL3, suggesting that METTL3 is of significance during the progression of BCa.

m⁶A modification regulates RNA expressions by modulating splicing and translation efficiency. IGF2BP2 could affect the stability of m⁶A-modified mRNAs [18,19]. It has been reported that IGF2BP2 can bind to the m⁶A-modified mRNAs for extending its half-life, thus affecting mRNA translation [20,21]. Our findings uncovered that METTL3 was able to exert m⁶A modification on SOX2 mRNA. Meanwhile, IGF2BP2 was capable of binding m⁶A-modified SOX2 mRNA, thereafter regulating its protein level.

The transcription factor SOX2 is a member of the SRY-related HMG-box (SOX) family, which contributes to cell fate determination [22-24]. It has been reported that SOX2 is upregulated in mouse squamous skin tumor CSCs and involved in maintaining tumor cell stemness [25]. In addition, SOX2 is reported to be regulated by the shh

signaling and affects CSC in non-small cell lung cancer [26]. This experiment found that in BCa cells, METTL3 maintained the stemness of BCSCs by regulating SOX2, thus influencing the malignant level of BCa.

Conclusions

METTL3 is upregulated in BCa, and it promotes BCa progression through mediating m⁶A modification on SOX2 mRNA. METTL3-mediated m⁶A modification can be used as a therapeutic target for BCa.

Conflict of interests

The authors declare no conflict of interests.

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