Gene 722 (2020) 144076

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

N6-methyladenosine METTL3 promotes the breast cancer progression via targeting Bcl-2

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ARTICLE INFO

Keywords:
N6-methyladenosine
m6A
METTL3
Breast cancer
Bcl-2

ABSTRACT

N6-methyladenosine (m6A) is the most prevalent internal modification in mammalian mRNAs and methyltransferase-like 3 (METTL3) is a vital methyltransferase in m6A modification. Here, this study tries to discover the regulatory role of METTL3 and its mechanism in the breast cancer tumorigenesis. Results found that METTL3 was up-regulated in the breast cancer tissue and cells. In vivo and vitro, METTL3 knockdown could decrease the methylation level, reduce the proliferation, accelerate the apoptosis and inhibited the tumor growth. Moreover, we found that Bcl-2 acted as the target of METTL3, thereby regulating the proliferation and apoptosis of breast cancer. This study could reveal the potential mechanism of m6A modification in the breast cancer tumorigenesis, providing potential drug targets in the treatment.

1. Introduction

Breast cancer is the most common death-related malignant tumor for women worldwide, accounting for the leading cause of gynecological oncology in both developed and underdeveloped countries(Davari et al., 2017; Khan et al., 2017). On clinical and practical level, the large proportion of diagnosed or death case caused by breast cancer might give rise to the public health issues and economic pressures (Laderian and Fojo, 2017; Tian et al., 2018b). Although the emerging therapeutic methods for breast cancer have been developed in decades, including surgery and chemotherapy, there are about 30% of patients are confronted with the relapse or metastasis(Tian et al., 2018a). Therefore, the problems we face during the therapy are still imperious.

N6-methyladenosine (m6A) modification is one of the most prevalent internal modification in mammalian mRNAs and eukaryotes that occurred in the consensus motif (Zhang et al., 2019). In 1970s, m6A was firstly described without any special attention. In recent years, more and more researchers arouse great interest for the m6A modification in cancer research or non-cancer research (Wu et al., 2018). Increasing evidences indicated the critical roles of epigenetics modification for cancer as well as m6A, such as noncoding RNA, chromatin remodeling and histone modification (Wu et al., 2019a; Zhao et al., 2019). There are three major functional enzymes participate in the m6A modification, including methyltransferase (METTL3, METTL14, WTAP) (Liao et al., 2019), demethylase (FTO, ALKBH5) (Huttelmaier et al., 2019) and methylated reader proteins (YTHDF1/2/3, HNRNPA2B1, IGF2BP) (Lan et al., 2019).

Methyltransferase-like 3 (METTL3) is a subgroup of methyltransferase family, acting as the N6-adenosine methyltransferase. In multiple human cancers, METTL3 has been reported to exert critical roles. In this research, we found that METTL3 was up-regulated in the breast cancer tissue and cells. And, METTL3 could install the methylation of Bcl-2 and promote its translation, thereby regulating the proliferation and apoptosis of breast cancer.

2. Materials and methods

2.1. Clinical tumor and normal tissues collection

The breast tumor tissues and their matched normal tissue (30 pairs) were obtained from the Renji Hospital of Shanghai Jiao Tong University. Moreover, the experimental projects were approved by the Ethics Committee board of this hospital. During the surgical excision, the breast cancer tissue and adjacent normal tissue were obtained and then stored in the liquid nitrogen. All these enrolled specimens were accordant this standard.

Abbreviations: METTL3, methyltransferase-like 3; m6A, N6-methyladenosine

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https://doi.org/10.1016/j.gene.2019.144076
Received 15 June 2019; Received in revised form 21 August 2019; Accepted 22 August 2019
Available online 24 August 2019
0378-1119/

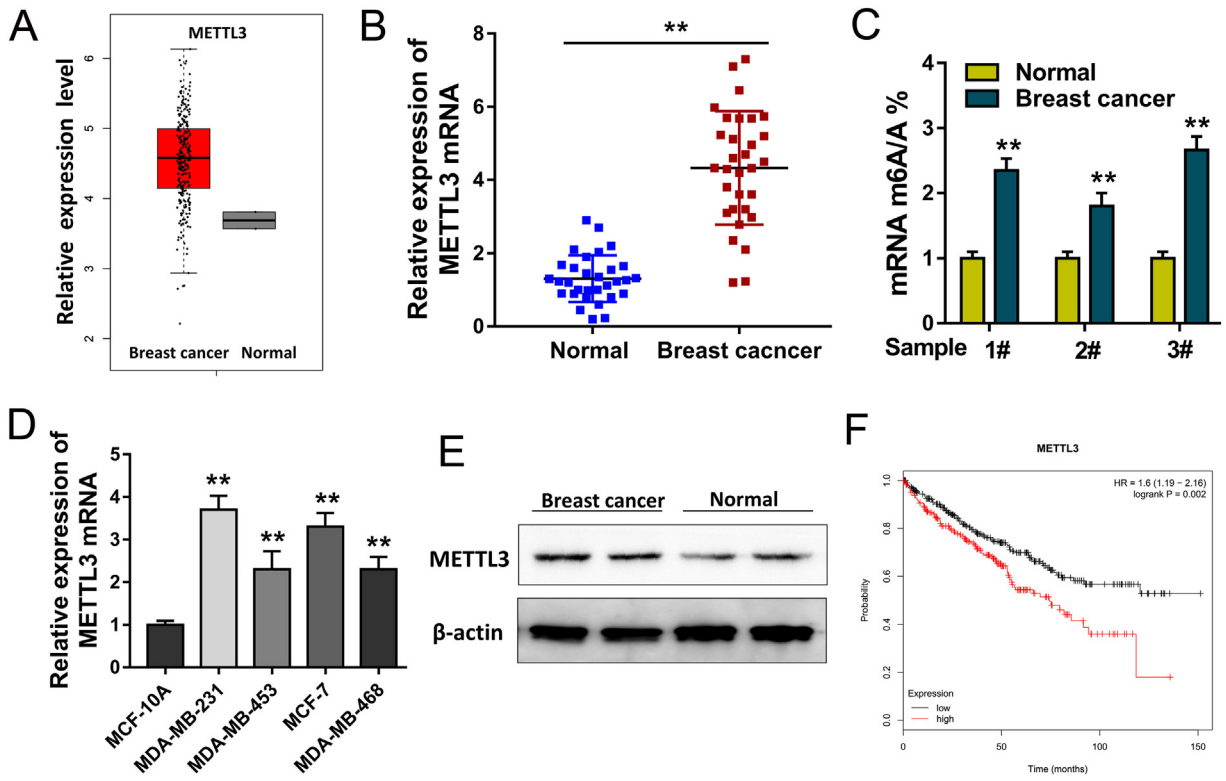

Fig. 1. METTL3 is up-regulated in the breast cancer tissue and cells. (A) The expression of METTL3 analyzed by the bioinformatics tools (GEPIA, http://gepia.cancer-pku.cn/). (B) METTL3 mRNA was analyzed by RT-PCR in the enrolled breast cancer individuals and controls. (C) mRNA m6A quantitative analysis showed that the m6A level. (D) METTL3 protein level in the breast cancer specimens and normal controls. (E) Western blot analysis revealed the METTL3 protein. (F) The prognosis analysis using the Kaplan-Meier test showed the r survival rate and outcome of breast cancer patients. $^{**}p$ value ≤ 0.01.

2.2. Cells culture

Breast cancer cell lines (MDA-MB-231, MCF-7, MDA-MB-468, MDA-MB-453) and normal human breast epithelial cell (MCF-10A) were obtained from American Type Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Corning, USA) with 10% fetal bovine serum (FBS, Gibco, USA) and at 5% CO_2 cell culture under 37 °C.

2.3. Cell transfection

The short hairpin RNA targeting METTL3 and Bcl-2 overexpressing plasmids were provided by Ribobio (Guangzhou, China) and then respectively or co-transfected into breast cancer cells. The transient transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4. Total RNA extraction and RT-PCR

Total RNAs were extracted by Trizol (Thermo Fisher, USA) and the complementary DNA (cDNA) was synthesized using the SuperScript™ III First-Strand Synthesis System DNA and PrimeScript RT reagent Kit (RR036A, Takara, Japan). Reverse-transcription qPCR was performed using SYBR Premix Ex Taq (TaKaRa) in on7500 system. GAPDH functioned as the normalized internal control. The primers were presented in the supplementary Table S1.

2.5. RNA m6A quantification

Total RNAs were isolated by TRIzol (Thermo Fisher, USA) according to the manufacturer's instructions. The relative content of m6A in the total RNA was measured using the EpiQuik m6A RNA Methylation Quantification Kit (Colorimetric) (P-9005, Epigentek, USA) according to the manufacture's instruction. In brief, 200 ng RNAs were administrated with the solution containing the antibody. The m6A levels were quantified using the colorimetrical analysis via absorbance at 450 nm.

2.6. Western blot

Protein component of cells or tumor tissue were lysed with RIPA lysis buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5, Triton, EDTA and glycerophosphate) with protease inhibitor. The concentration was measuredd using BCA protein kit (Pierce, USA). Then, cell extractions were separated by 12% SDS-PAGE and electro-blotted to PVDF membrane (Amersham Pharmacia, Germany). The membrnes were incubated with primary antibody (anti-METTL3, Abcam, ab195352, anti-Bcl-2, ab32124) overnight on a rocker at 4 °C. Goat anti-mouse GAPDH (Sigma, USA) followed by enhanced chemiluminescence (ECL, Amersham Pharmacia, USA).

2.7. CCK-8 assay

The cellular proliferation potential of breast cancer was measured using the CCK-8 agent (CCK-8 assay kit, Dojindo Japan). Cells were seeded out in 96-well plates and incubated with 8 µl of CCK-8 solution according to the manufacture's protocols. Absorbance was determined at 450 nm.

2.8. Flow cytometry

Apoptosis was measured with flow cytometry using Annexin apoptosis detection kit (KeyGen, China) based on the manufacturer's protocol. In brief, the cells were washed with cold PBS for twice and then resuspended in Binding Buffer (100 µl) and then stained with FITC

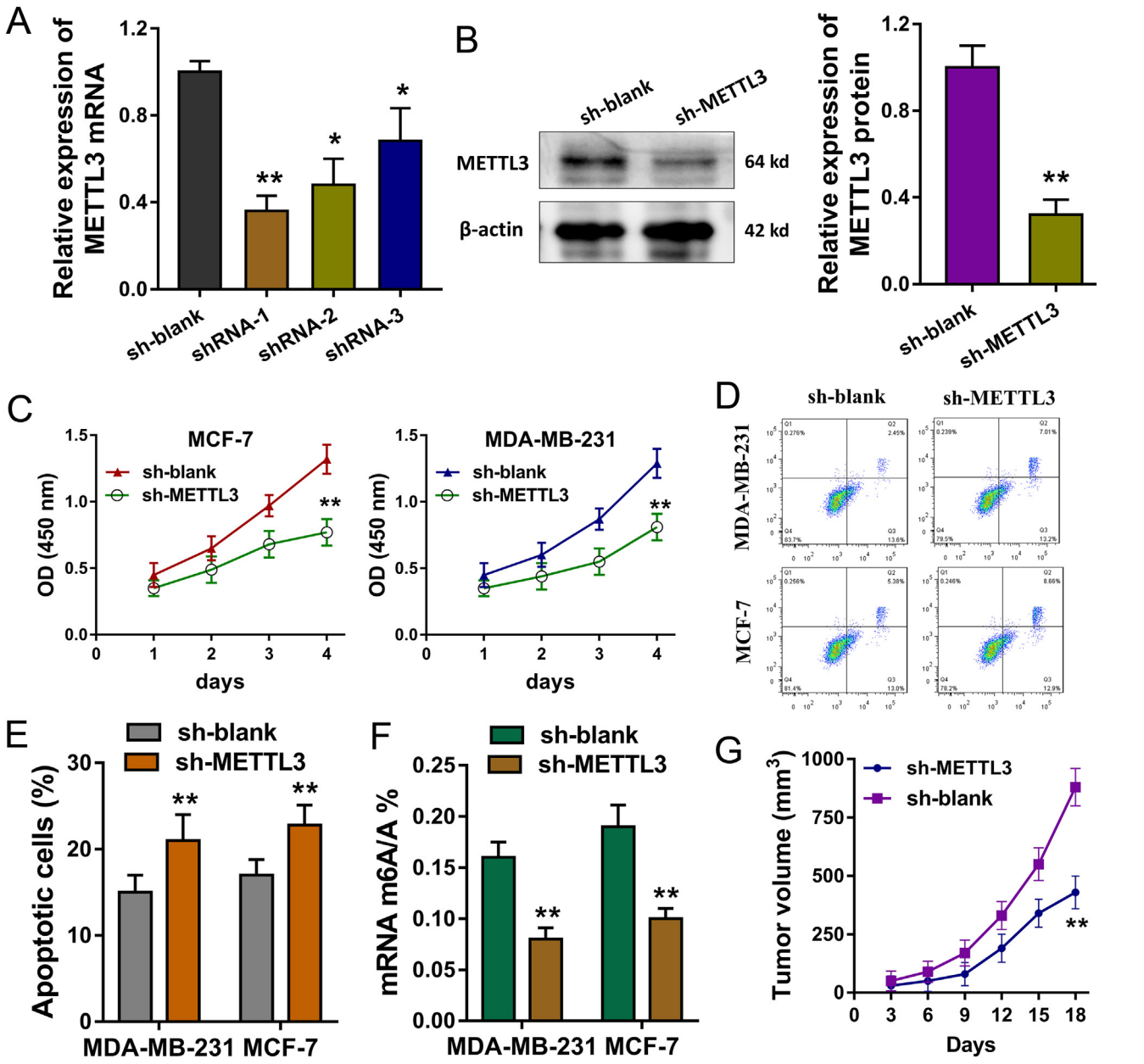

Fig. 2. Silencing of METTL3 represses the proliferation and induced the apoptosis of breast cancer cells. (A) Oligonucleotides short hairpin RNA (shRNA) targeting the METTL3 were transfected into breast cancer cells to silence the METTL3 expression. (B) Western blot analysis indicated the METTL3 protein level. (C) CCK-8 analysis indicated the proliferative ability of breast cancer cell lines (MDA-MB-231, MCF-7) after the METTL3 silencing or not. (D, E) Apoptosis analysis using the flow cytometry illustrated the apoptotic cell rate of breast cancer cells. (F) The mRNA m6A quantitative analysis indicated the m6A quantitation when the METTL3 was silenced no not. (G) In vivo mice heterotransplantation assay presented the tumor growth in vivo transplantation with METTL3 silencing. **p value ≤ 0.01.

Annexin V (5 μl) and propidium iodide (PI, 5 μl). The quantitatively results were analyzed by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) as percentage, including early apoptosis and late apoptosis cells.

2.9. In vivo tumor growth

The in vivo animal procedures were approved by the Institutional Committee of Renji Hospital of Shanghai Jiao Tong University. Two group of (MDA-MB-231 cells, METTL3 silencing group and control group, were injected into the flank of Balb/C mice. The length and width were recorded every three days. Three weeks later, the e xeno-graft neoplasm was incised and weighed. The volume was calculated according to the formula: (width/2)2 × (length/2) × 4π/3.

2.10. Statistical analysis

GraphPad Prism vision 6.0 and SPSS vision 19.0 software were used for the statistical analysis. The data value is presented as the mean ± standard error. The analysis was performed using two-tailed Student's *t*-test r one-way ANOVA. Statistical significance was considered as *P*-value < 0.05.

3. Results

3.1. METTL3 is up-regulated in the breast cancer tissue and cells

In the first step, we analyzed the expression of METTL3 using the bioinformatics tools (GEPIA, http://gepia.cancer-pku.cn/), suggesting the over-expression of METTL3 in the breast cancer cohort (Fig. 1A).

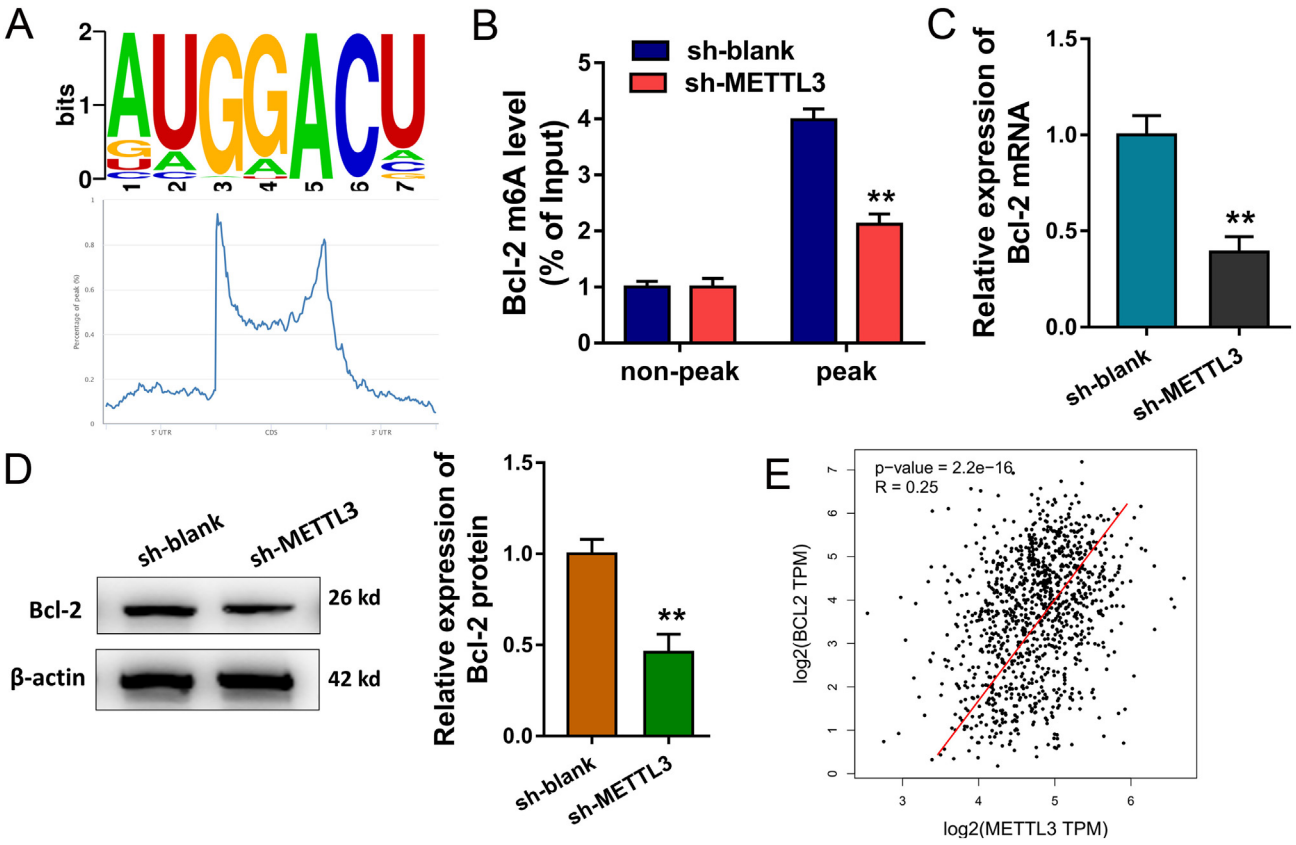

Fig. 3. Bcl-2 is the target of METTL3. (A) The m6A sequences motif from the top m6A peaks identified that Bcl-2 might act as the downstream target of METTL3 in the breast cancer. (B) Gene-specific m6A-qPCR revealed the Bcl-2 m6A level.
The non-peak region acted as the control. (C) RT-PCR illustrated the Bcl-2 mRNA with the silencing of METTL3. (D) Western blot analysis showed the Bcl-2 protein with the silencing of METTL3. (E) The data from the TCGA dataset revealed the positive correlation within METTL3 and Bcl-2. **p value ≤0.01.

Besides, we measured the expression of METTL3 mRNA in the enrolled breast cancer individuals and found that METTL3 mRNA was remarkedly up-regulated in the breast cancer group (Fig. 1B). The mRNA m6A quantitative analysis showed that the m6A level was increased in the breast cancer tissue as compared to normal samples (Fig. 1C). In the breast cancer cells, METTL3 mRNA levels were increased correlated to the normal controls (Fig. 1D). Western blot analysis revealed that METTL3 proteins were highly expressed in the breast cancer specimens in keeping with the cellular abundance (Fig. 1E). The prognosis analysis using the Kaplan-Meier test showed that the high-expression of METTL3 was correlated with the poor survival rate and outcome (Fig. 1F). Therefore, METTL3 is up-regulated in the breast cancer tissue and cells.

3.2. Silencing of METTL3 represses the proliferation and induced the apoptosis of breast cancer cells

The oligonucleotides short hairpin RNA (shRNA) targeting the METTL3 were transfected into breast cancer cells to silence the METTL3 expression (Fig. 2A). Western blot analysis indicated that the METTL3 protein level was remarkedly decreased after the transfection (Fig. 2B). CCK-8 analysis indicated that METTL3 silencing could repress the proliferative of breast cancer cell lines (MDA-MB-231, MCF-7) (Fig. 2C). Apoptosis analysis using the flow cytometry illustrated that METTL3 silencing accelerated the apoptotic cell rate of breast cancer cell lines (MDA-MB-231, MCF-7) (Fig. 2D, E). The mRNA m6A quantitative analysis indicated that the m6A quantitation was decreased when the METTL3 was silenced (Fig. 2F). In vivo mice heterotransplantation assay presented that the METTL3 silencing reduced the tumor growth in vivo transplantation (Fig. 2G). Overall, the silencing of METTL3 represses the proliferation and induced the apoptosis of breast cancer cells.

3.3. Bcl-2 is the target of METTL3

Previously research has reported that Bcl-2 might mediated by the METTL3 in lymphocytic leukemia (Vu et al., 2017). The identification of the downstream target of METTL3 in the breast cancer was performed using the m6A sequences motif from the top m6A peaks (Fig. 3A). Gene-specific m6A-qPCR revealed the Bcl-2 m6A level was higher in the peak region, and which was reduced when the METTL3 was silenced (Fig. 3B). RT-PCR illustrated that the silencing of METTL3 could decrease the Bcl-2 mRNA (Fig. 3C). Western blot analysis showed that silencing of METTL3 could decrease the Bcl-2 protein (Fig. 3D). The data from the TCGA dataset revealed that Bcl-2 was positively correlated with METTL3 in the breast cancer individuals (Fig. 3F). Overall, the data suggests that Bcl-2 is the target of METTL3.

3.4. METTL3 target the Bcl-2 to regulate breast cancer proliferation and apoptosis

The previous data had revealed that METTL3 silencing could decrease the Bcl-2 mRNA expression. In the further rescue assay, we found that the co-transfection of Bcl-2 overexpression plasmid and METTL3 silencing could rescue the Bcl-2 mRNA expression (Fig. 4A). Of course, Bcl-2 overexpression plasmid transfection could up-regulate the Bcl-2 mRNA (Fig. 4B). The apoptosis analysis and proliferative CCK-8 assay showed that the co-transfection of Bcl-2 overexpression plasmid and METTL3 silencing could rescue proliferation and apoptosis of breast cancer (Fig. 4C, D). Interesting, the regulation of METTL3 on Bcl-2 could explain that METTL3 promote the methylation of Bcl-2 and then

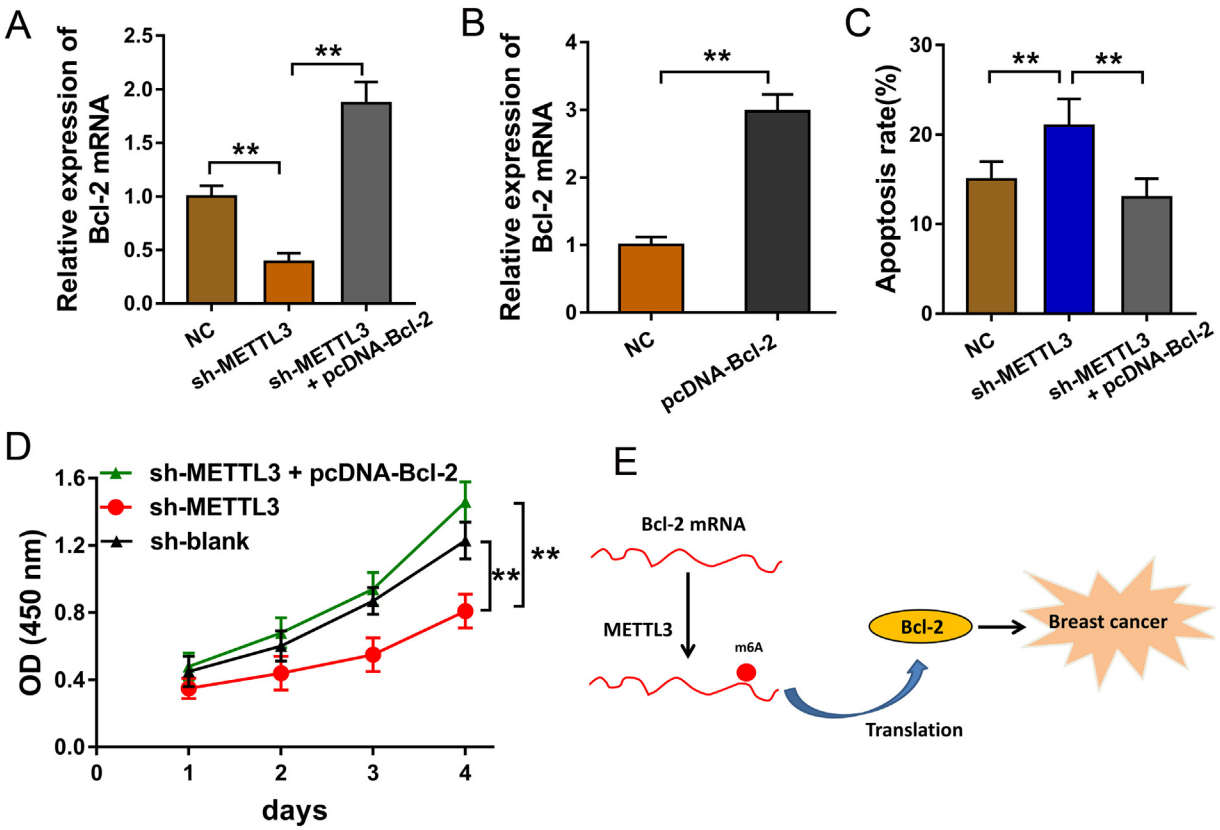

Fig. 4. METTL3 target the Bcl-2 to regulate breast cancer proliferation and apoptosis. (A) RT-PCR showed that the co-transfection of Bcl-2 overexpression plasmid and METTL3 silencing could rescue the Bcl-2 mRNA expression. (B) RT-PCR showed the Bcl-2 mRNA level after Bcl-2 overexpression plasmid transfection. (C) The apoptosis analysis detected by flow cytometry. (D) Proliferative CCK-8 assay. (E) The schematic diagram showed the mechanism for the m6A modification of METTL3 on Bcl-2. **p value ≤ 0.01.

promote its translation, which was proposed as previously reported literature (Vu et al., 2017). The schematic diagram showed the mechanism for the m6A modification of METTL3 on Bcl-2 (Fig. 4E).

4. Discussion

N6-Methyladenosine (m6A) is firstly identified in the 1970s, acting as the most prevalent internal modification for mammalian mRNAs (Choe et al., 2018). Emerging evidence support that m6A participate in the multiple disease genesis and tumorigenesis, and its pivotal roles draw great attention (Mendel et al., 2018). More and more theories indicate that the m6A modification could significantly regulate the tumorigenesis of human cancers, which might provide a potential therapeutic target.

There are > 7600 mRNA transcripts which could be detected with the m6A sites (Zhou et al., 2019). Interestingly, the m6A consistently occurs at the consensus RRACH motif, among which, the R might be G or A, and the H might be A, C or U (Wu et al., 2019b; Yang et al., 2019). Based on current experimental evidence, m6A modification could regulate the mRNA stability, translation and splicing process. There are three types of m6A related proteins including writers (methyltransferase, METTL3, METTL14 and WTAP), erasers (FTO, ALKBH5) and readers (YTHDFs) (Deng et al., 2018; Tong et al., 2018). Among these critical enzymes, METTL3 has been found to regulate series of human cancers, such as breast cancer (Cai et al., 2018), gastric cancer (Lin et al., 2019) and so on.

In this research, we found that METTL3 was significantly up-regulated in the breast cancer tissue and cells, which was in accord with the previously reported literature. Besides, the m6A level in the breast cancer tissue is also increased as compared to normal controls. The silencing of METTL3 could repress the proliferation and accelerate the apoptosis of breast cancer cells. In vivo mice assay showed that silencing of METTL3 inhibited the tumor growth. Moreover, the silencing of METTL3 could also decrease the m6A level, suggesting the regulatory role of METTL3 for the m6A in the breast cancer. In this finding, we discovered the oncogenic functions of METTL3 for breast cancer, which is consistent with existed literature and might trigger another oncogenic mechanism for the oncogenesis.

Previously finding indicated that METTL3 could promote the translation process of several target genes in acute myelocytic leukemia, including MYC, Bcl-2 and PTEN (Vu et al., 2017). Therefore, we reasoned that METTL3 might target Bcl-2 to contribute the breast cancer progression. The m6A sequences motif from the top m6A peaks suggested that Bcl-2 might act as the downstream target of METTL3. Series of experiments confirmed that METTL3 directly installed the methylation of Bcl-2 at the 3'-UTR. Moreover, the expression levels of METTL3 and Bcl-2 was positively correlated. The con-transfection of Bcl-2 overexpression could reverse the inhibition caused by the METTL3 silencing. In breast cancer, m6A demethylase FTO is up-regulated and the FTO mediate the BNIP3 mRNA degradation through its m6A demethylation at the 3'-UTR via YTHDF2 independent mechanism (Niu et al., 2019). Because m6A is the most abundant messenger RNAs modification in eukaryotic cell, the important roles of m6A is found and realized in many bioprocesse. For example, METTL3 enhance the autophagic flux via TFEB-dependent manner, and TFEB regulates the METTL3 expression and ALKBH5 in opposite directions, forming a negative feedback loop (Song et al., 2019).

In conclusion, we found and confirmed the critical roles of METTL3 in the breast cancer. Mechanically, we found the regulatory function of METTL3 via targeting Bcl-2 to modulate the proliferation and apoptosis. This finding might provide a novel insight for the m6A modification in the breast cancer.

Declaration of competing interest

All authors declare no conflicts of interest.

Acknowledgement

No.

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