METTL3 Depletion Contributes to HR+/HER2- Breast Cancer Progression and Drug Resistance via m6A Modification of Constituents of the CDKN1A/EMT and BAX/caspase-9/-3/-8 Signalling Pathways

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Research

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Abstract

Background

Chemotherapy is an important strategy for the treatment of hormone receptor positive / human epidermal growth factor receptor 2 negative (HR+/HER2-) breast cancer (BC), but this subtype has a low response to chemotherapy. Growing evidence indicates that N6-methyladenosine (m6A) is the most common RNA modification in eukaryotic cells and that methyltransferase-like 3 (METTL3) participates in tumour progression in several cancer types. Therefore, exploring the function of METTL3 in HR+/HER2- BC initiation and development is still significant.

Methods

mRNA and protein expression levels were analysed by quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting, respectively. Cell proliferation was detected by CCK-8 assays, cell migration was analysed by wound healing assays, and apoptosis was analysed by TUNEL assays. Finally, m6A modification was analysed by methylated RNA immunoprecipitation (MeRIP).

Results

Chemotherapy-induced downregulation of the m6A modification is regulated by METTL3 in HR+/HER2- BC. METTL3 knockdown in MCF-7/T47D cells weakened the drug sensitivity of HR+/HER2- BC cells by promoting tumour proliferation and metastasis and inhibiting apoptosis. Mechanistically, CDKN1A was subsequently recognized as a downstream target of METTL3 that activates the AKT pathway and promotes epithelial-mesenchymal transformation (EMT). Moreover, the RNA level of BAX was decreased due to a lower level of m6A modification mediated by METTL3, and apoptosis was inhibited by inactivation of caspase 3/9/8.

Conclusion

METTL3 regulates the proliferation, migration, and drug sensitivity of HR+/HER2- BC via activation of the CDKN1A/EMT and m6A-BAX/caspase 9/3/8 signalling pathways, which suggests its role as a potential biomarker for predicting the prognosis of patients with HR+/HER2- BC.

Background

Globally, breast cancer (BC) is a highly heterogeneous disease with complex molecular bidirectional crosstalk between hormone receptors (HRs) and human epidermal growth factor receptor 2 (HER2)(1, 2). Approximately 70% of patients have BC that is HR positive and HER2 negative (HR+HER2-)(3). Although endocrine-based therapies (aromatase inhibitors [AIs] and/or anti-oestrogen treatments with or without
ovarian suppression) are the gold standard of treatment of BC and the backbone of adjuvant therapies for these patients with significantly decreased risk of recurrence and death(4), up to 20% of patients will eventually relapse(5, 6). Single-agent chemotherapy is an essential treatment option for endocrine-resistant or treatment-refractory disease(7). However, response rates to these therapies are low. Reported progression-free survival (PFS) ranges from 4.0 to 6.3 months with second-line chemotherapy and from 2.4 to 5.5 months with third-line chemotherapy(8). Similarly, the association of pathological complete response (pCR) with disease-free survival (DFS) or overall survival (OS) in HR+/HER2− BC following neoadjuvant systemic therapy is relatively low compared to that of the other two subtypes of BCs(9). Thus, there is an imperative need to further improve our insight into the tumour biology and the tumour cell response to chemotherapy for HR+/HER2− BC.

$N^6$-methyladenosine (m6A) is the most prevalent messenger RNA (mRNA) modification in eukaryotes and is added to mRNA molecules by the $N^6$-adenosine methyltransferase complex, which consists of methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14) and Wilms tumour 1 associated protein (WTAP)(10). METTL3 and METTL14 are two active methyltransferases that form a heterodimer to catalyse m6A RNA methylation, while WTAP interacts with this complex and substantially affects mRNA methylation(11). Two m6A demethylases fat mass and obesity-associated (FTO) and AlkB homolog 5 (ALKBH5) have been discovered since 2011, revealing the dynamic nature of m6A modification(12). Some cellular proteins have been found to preferentially bind m6A-modified RNA, whereas others been characterized to specifically recognize m6A-modified mRNA and accelerate the decay of the mRNA(13). These results indicated that this chemical modification is common and important in a variety of biological processes.

With the elucidation of the mechanisms involved in m6A modification, a recent report described the role of the m6A modification in multiple tumours(14). Although studies on the function of m6A in BC are still in their early stages, there is growing evidence showing that m6A plays a critical role in many aspects of BC, including tumorigenesis(15), metastasis(16), prognosis(17), and treatment resistance(18). Reduced expression of METTL3 promotes metastasis of BC by increasing COL3A1 expression(19). Hypoxia stimulates ALKBH5, which stabilizes NANOG mRNA and induces a phenotype associated with BC stem cells (BCSCs) and lung metastasis(20, 21). YTHDF3 promotes BC metastasis to the brain by inducing m6A-enriched gene translation(16). Moreover, m6A modification patterns have therapeutic implications and correlate with drug resistance. HNRNPA2B1 and METTL3 overexpression in MCF-7 cells reduces their sensitivity to 4-hydroxytamoxifen and/or fulvestrant(22, 23). In triple-negative BC (TNBC) cells, IGF2BP3 promotes chemoresistance to doxorubicin (DOX) and mitoxantrone by regulating ABCG2 expression(24). Therefore, it is possible to determine why HR+/HER2− BC is insensitive to chemotherapy treatment by further exploring the role of m6A modification in this subtype of BC.

In this study, we investigated the potential effect of m6A methylation on the sensitivity of HR+/HER2− BC to chemotherapy. Our data revealed that chemotherapy decreased the levels of the m6A modification, which was dependent on METTL3 expression. We demonstrated that METTL3 facilitates HR+/HER2− BC progression via its downstream target cyclin-dependent inhibitor kinase 1A (CDKN1A), which mediates
epithelial-mesenchymal transition (EMT). In addition, METTL3 regulated BAX/caspase3/8/9 signalling in an m6A-independent manner. Overall, our results suggested that METTL3 plays multifunctional roles in the progression of HR+/HER2− BC, indicating that METTL3 is a promising biomarker for predicting the efficacy of chemotherapy as well as a potential therapeutic target for reversing chemotherapy resistance in HR+/HER2− BC.

Methods

Human BC tissues and cell lines

Thirteen pairs of primary BC tissues collected before and after treatment were obtained from the Second Xiangya Hospital of Central South University (Hunan, China) from August 2020 to March 2021. All individuals with BC were diagnosed for the first time, only received chemotherapy prior to surgery, and had histologically confirmed BC. All patients provided written informed consent, which was conducted in accordance with the Declaration of Helsinki. BC cell lines (MCF-7 and T47D) were obtained from the Shanghai Type Culture Collection of the Chinese Academy of Sciences and were grown in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 1% penicillin/streptomycin (Shanghai, Beijing, China). All cells in this study were incubated in 37°C incubators with 5% carbon dioxide and routinely tested for mycoplasma.

Cell transduction

Stable knockdown and overexpression of METTL3 were achieved with lentiviral-based delivery of short-hairpin RNA (shRNA) and overexpression vectors, respectively. The shRNA sequences were subcloned into a lentiviral expression vector containing GFP by Shanghai Genechem Co., Ltd. (Shanghai, China). Lentiviral transduction was performed according to the manufacturer's instructions. All constructed vectors were verified by DNA sequencing.

Western blotting

Total proteins from cell lines and tissues were extracted with RIPA buffer and then quantified by BCA analysis. Subsequently, 20 µg of total protein per sample (10 µL per lane) was separated using sodium lauryl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide gel) before the proteins were transferred to a PVDF membrane. After incubation with primary antibodies overnight, the membranes were then incubated with secondary antibody. Finally, target protein bands were detected using a chemiluminescence system. The antibodies used targeted the following proteins: AKT (Cell Signaling #9272s), Caspase 9 (Proteintech 10380-1-AP), Caspase 3 (Proteintech 66470-2-Ig), Caspase 8 (Proteintech 66093-1-Ig), p-ATK (Cell Signaling #4060), BAX (Proteintech 60267-1-Ig), Vimentin (Proteintech 10366-1-AP), N-cadherin (Proteintech 22018-1-AP), E-cadherin (Proteintech 20874-1-AP), GAPDH (Signalway Antibody #21612), and METTL3 (ABclonal A8370).

Quantitative real-time polymerase chain reaction (qRT-PCR)
Total RNA from cell lines and tissues was isolated using TRIzol reagent (Thermo Fisher Scientific, Beijing, China). A PrimeScript RT kit (Thermo Fisher Scientific, Beijing, China) was used for cDNA synthesis. Real-time quantitative PCR analysis was performed using a SYBR Premix Kit (Abclonal, Wuhan, China). Each sample was run in triplicate, and the expression levels were normalized to those of GAPDH using relative quantitative methods. All PCR primers (Well Biological Science, China) are listed as follows:

GAPDH-F: ACAGCCTCAAGATCATCAGC
GAPDH-R: GGTCATGAGTCTTCCACGAT
METTL3-F: TTGTCTCCAACCTTCCGTAGT
METTL3-R: RCCAGATCAGAGGTGGTGTAG
CDKN1A-F: CAAGCTCTACCTTCCCACGG
CDKN1A-R: TCGACCCTGAGGTCTCCAG
BAX-F: ACTAAAGTGCCCGAGCTGA
BAX-R: ACTCCAGCCACAAAGATGGT

**Cell proliferation assay**

Cell proliferation and cytotoxicity assay kits (Dingguo, Beijing, China) were used to assess cell viability. Cells were seeded into 96-well plates at a density of $1 \times 10^3$ cells per well and cultured in an incubator (37°C with 5% CO2) for 24 h, 48 h, and 72 h, after which cell proliferation was examined on a microplate reader by measuring the absorbance at a wavelength of 450 nm. To assess colony formation, cells were seeded into 6-well plates at a density of $1 \times 10^3$ cells per well and cultured in an incubator (37°C with 5% CO2). After 14 days, the cells were fixed and stained with Giemsa stain, modified solution (Sigma), and colonies containing 50 or more cells were counted.

**Cell cycle analysis**

For cell cycle analysis, cells were seeded in 6-well plates at a density of $2–3 \times 10^5$ per well and grown to ~70% confluence. Cells were then harvested and suspended in complete medium. The cell suspension was centrifuged at 1300 rpm at 4°C, washed once in D-Hanks buffer, counted and resuspended to a density of $3–6 \times 10^6$ cells/ml. Ice-cold 70% ethanol was added dropwise to fix the cells, which were then centrifuged, washed once with D-Hanks, stained with 20 µg/ml propidium iodide (Sigma, P4170) in D-Hanks containing 50 µg/ml RNase A (Fermentas, EN0531) for 1 h at room temperature and analysed using flow cytometry.

**Wound healing assay**

For the wound healing assay, cells were seeded and cultured until a 90% confluent monolayer was formed. Cells were then scratched by a sterile pipette tip and treated as indicated in the text in FBS-free
medium. Cell migration distances into the scratched area were measured in 10 randomly chosen fields under a microscope.

**TUNEL assay**

A TUNEL kit (Roche) was used according to the manufacturer’s instructions. In brief, cells were fixed with 4% paraformaldehyde, permeated with 0.1% Triton X-100 in PBS, incubated with 50 µl of TUNEL reaction mixture for 1 h at 37°C, and then washed with PBS 3 times. A total of 1,000 cells were counted, and the percentage of apoptotic cells was quantified.

**Immunohistological staining**

Fresh tumour tissues were excised from nude mice and fixed with 4% paraformaldehyde. Then, the sections were dehydrated with a graded alcohol series, cleared in xylene, embedded in paraffin, and sliced into sections with a microtome at a thickness of approximately 4 mm. After more treatments with xylene and an alcohol gradient, the sections were dewaxed and hydrated. Ki-67 antibody was added and then incubated overnight, and secondary antibody was incubated for 1 h. Diaminobenzidine (DAB) was used for colour development, and the slides were stained with haematoxylin before they were mounted in neutral resin. With a common microscope, 5 40× high-definition fields were randomly selected for counting the total number of cells and the number of Ki-67-positive cells (which have brown nuclei).

**In vivo tumour xenograft model**

Eight nude female BALB/c mice (4 weeks old) were used for tumour formation experiments. After METTL3-overexpressing and control MCF-7 cells were digested into a single-cell suspension, they were washed twice with PBS to remove the residual medium, and 1X10^7 cells were injected into the axilla of the left and right forelimbs of nude mice. The health of the nude mice and the growth of the tumours were observed every three days. Tumour volume was calculated with the following formula: V = (L × W^2)/2 cm³ (V, tumour volume; L, length; W, width). The in vivo experiments were performed in accordance with the guidelines for the use of laboratory animals.

**RNA m6A quantification, methylated RNA immunoprecipitation (MeRIP), and quantitative PCR**

The m6A content of 200 ng of RNA extracted from tissues and cell lines was measured by an EpiQuik m6A RNA methylation quantification kit (Epigentek, USA) according to the manufacturer's instructions. Immunoprecipitation of m6A-modified BAX and CDKN1A mRNA was performed using a Tiangen MeRIP m6A Kit (FP313, Tiangen, China) according to the manufacturer's protocol. m6A enrichment was analysed by qPCR with specific primers (Well Biological Science, China), and data were normalized to the input. Primer sequences were as follows:

BAX-Positive-F: AGGATCGAGCAGGGCGAAT

BAX-Positive-R: AGCTGCCACTCGGAAAAAGA
BAX-Negative-F: CCGAGTCACTGAAGCGACTG
BAX-Negative-R: ACGTGGGCGTCCCAAAGTAG
CDKN1A-Positive-F: TCTTCGGCCCAGTGGACA
CDKN1A-Positive-R: AGTCGAAGTTCCATCGCTCA
CDKN1A-Negative-F: TCCTCATCCCGTGTTCTCCT
CDKN1A-Negative-R: ACAAGTGGGGAGGAGGAAGT

**Bioinformatics analysis**

The expression profiles of m6A-related mRNAs were obtained from the GSE87455 and GSE763 datasets ([https://www.ncbi.nlm.nih.gov/geo/](https://www.ncbi.nlm.nih.gov/geo/)), which comprised 69 pairs of pre- and post-chemotherapy BC tissues and MCF-7 cells treated with DOX, respectively. Differential expression of m6A-related genes between the pre-treatment and post-treatment tissues were determined by R software ([http://www.r-project.org/](http://www.r-project.org/)). The association of METTL3 expression with patient OS was analysed with Kaplan–Meier Plotter ([http://kmplot.com/analysis/index.php?p=service&cancer=pancancer_rnaseq](http://kmplot.com/analysis/index.php?p=service&cancer=pancancer_rnaseq)) based on the TCGA data. Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes (DEGs) were performed using the DAVID database ([https://david.ncifcrf.gov/](https://david.ncifcrf.gov/)).

**Statistical analysis**

Experimental data are presented as the mean ± standard deviation (SD) and were analysed using GraphPad Prism 8.0 software. All in vitro results are representative of at least three independent trials. Two-group comparisons were assessed by the Mann-Whitney U test or Student’s t test, and paired t tests were employed for paired BC and corresponding chemo-only BC samples. A two-tailed p-value of 0.05 was considered statistically significant.

**Results**

**Chemotherapy-induced decreases in METTL3 expression correlated with a poor prognosis in HR+HER2- BC**

To identify the crucial regulator of m6A modification in the progression of HR+HER2- BC, we screened the expression profiles of m6A writers (METTL3, METTL14, and WTAP) and m6A erasers (FTO and ALKBH5) in the GSE87455 platform. The results of the analysis showed that METTL3 was reduced and FTO was significantly increased in the chemo-only group, whereas METTL14, WTAP and ALKBH5 expression levels showed no significant differences between the untreated specimens and chemo-only tumour specimens (Figure 1A). We also examined these m6A-related genes in the GSE763 dataset (Additional file 1A), and only the pattern of METTL3 expression was consistent with that in the GSE87455 cohort. Next, we
detected METTL3 expression in 13 pairs of pre- and post-chemotherapy HR+HER2- BC tumour tissues and observed that METTL3 expression was deficient in the post-chemotherapy tissues compared to the pre-treatment tissues (Figure 1B, C). In addition, the downregulation of METTL3 in tissues after chemotherapy was consistent with the expression pattern in the MCF-7/ADR cell line (Figure 1D). MCF-7 and T47D cell lines treated with chemotherapeutic drugs, including DOX (Figure 1E, F), paclitaxel (PTX) (Additional file 1B) and cisplatin (DDP) (Additional file 1C), corroborated these findings. RT-PCR and western blot confirmed that METTL3 expression was reduced at the mRNA and protein levels in the DOX-treated group. Furthermore, individuals with HR+HER2- BC and low METTL3 expression had a worse prognosis, and METTL3 expression was correlated with recurrence-free survival (RFS) (Figure 1G, H).

**METTL3 depletion attenuated the drug sensitivity of HR+/HER2- BC cells by promoting tumour proliferation and metastasis and inhibiting apoptosis**

We first detected the global m6A level in BC tissues and cell lines using an m6A RNA methylation quantification kit. The levels of m6A-modified RNA were remarkably higher in untreated BC tissues than in matched chemo-only tumour tissues (Figure 2A) and in control MCF-7 cells than in cells treated with chemotherapeutic drugs (Figure 2B-D). Similar results were observed in T47D cells treated with DOX, PTX and DDP (Additional file 2A). However, decreased global m6A levels were not observed in MDA-MB-231 cells, which are a TNBC cell line (Additional file 2B). To clarify the role of METTL3 in HR+HER2- BC tumorigenesis, we inhibited and upregulated METTL3 expression via transfection of shMETTL3 and LV-METTL3 vectors, respectively, in MCF-7 and T47D cells. The knockdown and overexpression efficiencies at the mRNA and protein levels were determined by RT-PCR and western blot in these two cell lines (Figure 2E, Additional file 2C). The levels of m6A modification were decreased upon METTL3 inhibition (Figure 2F). Notably, METTL3 knockdown obviously enhanced cell proliferation but reduced the sensitivity of MCF-7 cells to DOX as shown in the CCK-8 assays (Figure 2G, H). As anticipated, METTL3 knockdown enhanced colony formation, especially in the DOX-treated group (Figure 2I), whereas METTL3 overexpression evoked the opposite effects (Figure 2J). Furthermore, a cell cycle assay demonstrated that silencing METTL3 prominently increased the proportion of cells in S phase (Figure 2K). However, METTL3 upregulation did not obviously reduce the proportion of S phase cells (Figure 2L). Similarly, we also observed a protective effect of METTL3 in T47D cells (Figure 2SD-I).

Cancer metastasis is a critical characteristic of drug resistance in individuals with BC. A wound healing experiment was utilized to assess the effects of METTL3 on BC cell mobility. The results demonstrated that depletion of METTL3 induced, but overexpression of METTL3 weakened, MCF-7 and T47D cell migration in vitro (Figure 3A, Additional file 3A). Moreover, an increase in the number of apoptotic cells was observed among METTL3-overexpressing MCF-7 and T47D cells (Figure 3B, Additional file 3B). These results were further substantiated by the in vivo results. The same numbers of METTL3-overexpressing and control MCF-7 cells were injected into immunocompromised nude mice.
Overexpression of METTL3 suppressed tumour cell seeding, which led to a lower average tumour volume and weight (Figure 3C-E). Immunohistochemistry (IHC) confirmed that the expression of Ki-67, an indicator of tumour growth, was lower in the tumours derived from the METTL3-overexpressing cells (Figure 3F). Taken together, these findings suggest the anti-proliferative and migratory roles of METTL3 in BC cells.

**Multidimensional sequencing identifies CDKN1A as a downstream target of METTL3**

To better understand the possible mechanism by which METTL3 participates in HR+HER2- BC progression, we first performed DEG analysis of the GSE87455 and GSE763 datasets and identified 4049 and 1783 DEGs in these two cohorts, respectively. There were 498 overlapping genes altered by chemotherapeutic drugs in both datasets, among which cyclin-dependent kinase inhibitor 1A (CDKN1A), DNA topoisomerase II alpha (TOP2A) and cell division cycle 20 (CDC20) were filtered by the criteria $|\log_2 \text{FC}| \geq 1$ and p-value < 0.05 (Figure 4A). GO and KEGG enrichment analysis revealed that the DEGs were mostly linked to EMT, transcriptional activation of cell cycle inhibitor p21 and transcriptional activation of p53 responsive genes, indicating a regulatory role of METTL3 in the chemoresistance of HR+HER2- BC (Figure 4B, C). Because CDKN1A exhibited the most distinct differential expression, we chose this gene as a candidate target for subsequent experiments. RT-PCR and western blot experiments confirmed the positive regulation of METTL3 on CDKN1A at the mRNA and protein levels (Figure 4D; E). Additionally, MeRIP-qPCR using a m6A-specific antibody showed that the levels of m6A-modified CDKN1A were decreased in METTL3-silenced MCF-7 cells (Figure 4F). These data suggest that METTL3 regulates CDKN1A mRNA expression via m6A methyltransferase activity.

EMT plays a critical role in the drug resistance and metastasis of tumours. We also found that DOX induced a decrease in the expression of E-cadherin and an increase in the expression of N-cadherin and vimentin in MCF-7 and T47D cells (Figure 4G) indicative of EMT. Similarly, inhibition of METTL3 resulted in increased protein levels of N-cadherin and vimentin, whereas overexpression of METTL3 showed the opposite trends (Figure 4I, J). Furthermore, pseudopodia and cell spacing in MCF-7 cells were increased in response to METTL3 knockdown (Figure 4H). Collectively, these results suggested the importance of METTL3-mediated EMT signalling in chemoresistance.

**METTL3 regulates EMT in a PI3K/AKT signalling-dependent manner**

Despite the observed critical role of METTL3 in the EMT process, whether this activity is specifically attributed to the METTL3/CDKN1A axis needs to be further explored. HR+HER2- BC is insensitive to chemotherapy, which may be largely related to the mechanism of cellular feedback regulation to drug exposure. There is often a mutation in the PIK3CA gene in HR+HER2- BC, which plays a crucial role in regulating the PI3K/AKT pathway and drug resistance. However, the precise role of DOX-induced PI3K/AKT pathway activity has not been documented. Therefore, we first determined the effect of DOX intervention on the levels of p-AKT and total AKT. We observed increased levels of p-AKT in MCF-7 and...
T47D cells treated with DOX, as evidenced by the western blot experiments (Figure 5A, B). We also assessed the potential effects of METTL3 on the PI3K/AKT pathway. The data showed that METTL3 depletion resulted in upregulation of p-AKT levels. Conversely, MCF-7 cells with stable overexpression of METTL3 showed restrained p-AKT levels compared with those of the other groups (Figure 5C, D). Subsequently, we tested the influence of METTL3 and PI3K/AKT on the EMT pathway using the PI3K inhibitor LY294002. The data showed that the increased N-cadherin and p-AKT levels associated with METTL3 deficiency could be partially attenuated by treatment with LY294002 (Figure 5E), highlighting the essential role of PI3K/AKT in the METTL3-controlled EMT process. Additionally, we conducted bibliometric analysis and found that CDKN1A is one of the key molecules in the PI3K/AKT pathway (Additional file 3C). Genetic upregulation of CDKN1A also impaired the effects of METTL3 inhibition on p-AKT activation (Figure 5F). Our results clarify the critical role of CDKN1A and AKT in METTL3-mediated EMT progression in HR+HER2- BC.

**BAX activates caspase3/8/9 to facilitate METTL3-mediated tumour cell apoptosis**

We demonstrated that METTL3 promoted apoptosis (Figure 3B, Additional file 3B). Given the involvement of the apoptosis signalling pathway in tumour proliferation and metastasis, western blotting was implemented to visualize changes in the expression of apoptosis-related proteins following METTL3 knockdown or overexpression in MCF-7 and T47D cells. We found that increasing METTL3 expression elevated the levels of multiple apoptosis-related proteins, including caspase3, caspase8 and caspase9. By contrast, knockdown of METTL3 appreciably decreased the expression of these proteins (Figure 6A, B). BAX is an important protein that promotes apoptosis. As an upstream gene of caspase3, BAX regulates the activation of the caspase family. To further verify the relationship between METTL3 and apoptosis, the expression of apoptosis-related genes was detected at the mRNA and protein levels. We observed that increased METTL3 expression could facilitate the expression of BAX in MCF-7 cells and T47D cells (Figure 6C, D). Notably, MeRIP-qPCR confirmed the interaction between BAX mRNA and m6A in METTL3-depleted MCF-7 cell lines (Figure 6E). Therefore, we demonstrate that METTL3 increases BAX/caspase3/8/9 expression, promotes apoptosis and restrains tumorigenesis in HR+HER2- BC cells.

**Discussion**

In recent decades, substantial improvements have been made in therapeutic interventions for BC, which have increased the survival and quality of life of patients(25). Chemotherapy, as the broadest application of tumour treatment, remains the cornerstone of adjuvant therapy for BC and is widely used in BC patients with high metastatic burden and local advanced disease(26). Nevertheless, recent studies have indicated that the response rate of BC patients with the HR+/HER2- subtype to chemotherapy is low(27). Hence, the demand for elucidating the mechanism of HR+/HER2- BC insensitivity to chemotherapy is crucial. In this study, we first discovered that m6A modifications and METTL3 expression were inhibited by chemotherapy; thus, we evaluated the function of METTL3 in regulating HR+/HER2- BC progression, metastasis, and drug resistance. Based on our findings, we defined the METTL3/CDKN1A/EMT and
METTL3/BAX/caspase3/8/9 axes as novel pathways involved in a potential mechanism of HR+HER2-BC chemoresistance (Figure 6F).

m6A modification, one type of RNA epigenetic modification, has been identified on almost all types of RNAs and has been implicated in a variety of cellular processes, including mRNA stability, splicing, location, and translation, RNA-protein interactions, and pri-miRNA processes(28–33). An increasing number of studies have addressed the pathological significance of m6A dysregulation in human diseases, especially in cancers(28–30). The results of our current study showed that the overall level of m6A modification was significantly downregulated after chemotherapy in HR+/HER2− BC patients, and treatment of MCF-7 and T47D cells with DOX, PTX and DDP also resulted in a decrease in m6A modifications. However, the levels of m6A modification were not affected by drug intervention in MDA-MB-231 cells. Therefore, our results suggested that chemotherapy-induced changes in m6A levels are a biological difference between HR+HER2− BC and TNBC, especially in terms of responsiveness to chemotherapy. Various studies indicate that the m6A modification affects drug sensitivity by regulating ABC transporters either directly at the transcript level or via upstream signalling pathways(31). Recent studies also indicated that the m6A modification is involved in the maintenance of CSCs in tumours, leading to drug resistance and recurrence(32). It has also been shown that m6A modifications can affect the response of BC to endocrine therapy(22). However, there are few studies on the relationship between m6A and chemotherapy response. Therefore, considering the potential role of the m6A RNA modification in the development of chemoresistance, it is necessary to illustrate the relationship between these two phenomena.

METTL3, a key component of the N6-methyltransferase complex, has been reported to play an important role in many tumour types(33–38). Previous studies reported that METTL3 plays an oncogenic role in acute myeloid leukaemia through diverse downstream targets(38), whereas other studies suggested that either increased or decreased METTL3 expression could promote the self-renewal and tumorigenicity of glioma stem-like cells, respectively(37, 39). Regarding METTL3 in BC, data from the literature have suggested that METTL3 can promote BC progression by targeting Bcl-2, HBXIP or SOX2(36, 40, 41) and that METTL3 could promote adriamycin resistance by accelerating pri-miRNA-221-3p maturation(42). However, our results illustrated that chemotherapy-mediated depletion of METTL3 plays a significant protective role in tumour progression and drug tolerance. Reasonable explanations for these contradictory phenomena could be attributed to recognition by different m6A readers(43). We speculated that the m6A modification and METTL3 expression protect some critical genes from degradation or restrain the role of oncogenes by enhancing their recognition by “readers”. However, this hypothesis needs more study. In summary, the decreased METTL3 expression is secondary to chemotherapy, which is consistent with the clinical medication pattern, and HR+HER2− BC is the only BC subtype to exhibit this expression pattern. Therefore, METTL3 can be used as a biomarker to predict the sensitivity of HR+HER2− BC to chemotherapy and as a novel target for combination therapy to reverse chemotherapy resistance.

Our results further showed that METTL3 regulates the proliferation, apoptosis, migration and drug resistance of HR+/HER2− BC through multiple signalling pathways. On the one hand, METTL3 can affect
the m6A modification of BAX mRNA, thereby promoting activation of the pro-apoptotic caspase cascade and (consequently) apoptosis. Apoptosis is an important mechanism to mitigate the uncontrolled growth of tumour cells and is mainly regulated by the Bcl2 protein family(44). The Bcl2 protein family can be divided into two categories according to their functions: one plays a pro-apoptotic role and includes BAX and Bak, whereas the other plays an anti-apoptotic role and includes Bcl2. Both pathways promote caspase cascades that eventually lead to cell death(44). Our experiment found that METTL3 can promote the expression of BAX and the subsequent activation of caspase3, 8, and 9, leading to apoptosis.

On the other hand, downregulation of METTL3 can regulate CDKN1A expression to affect the EMT process and promote cell proliferation. CDKN1A is one of the key molecules involved in cell cycle progression and was first identified as a tumour suppressor(45). Later, it was found to be involved in pathways related to tumorigenesis and development, such as cell death, DNA replication/repair, gene transcription and cell motility(46). It is believed that the dual role of CDKN1A depends on its cellular localization(47). When in the nucleus, CDKN1A functions a tumour suppressor. However, when CDKN1A is concentrated in the cytoplasm, p53-impaired or p53-deficient cells may acquire carcinogenic properties, which may inhibit apoptosis and promote cell migration and proliferation(48). Studies have shown that miR-33b-3p can promote the survival and cisplatin resistance of A549 human lung cancer cells by targeting CDKN1A after DNA damage(49). It was also found that miR-520g mediated the resistance of colorectal cancer cells to 5-fluorouracil (5-FU) or oxaliplatin by downregulating of CDKN1A expression(50). These studies suggest that the presence of CDKN1A protects cancer cells from apoptosis after anti-cancer therapy. Therefore, in this study, the changes in CDKN1A expression were caused by chemical drugs, which may stimulate the translocation of CDKN1A protein from the nucleus to the cytoplasm, thereby activating downstream related pathways to reduce the sensitivity of cells to chemotherapy drugs; however, the specific mechanism is still unknown. In short, the mechanisms of interaction between cell signalling pathways and epigenetic elements are diverse and complex and merit further exploration and verification.

This study still has some shortcomings, such as the small sample size and lack of follow-up data. The direct intermolecular regulatory mechanism by which METTL3 affects tumour progression and survival was not clarified in detail and we will be further studied in the future.

Conclusion

Our data suggested the importance of chemotherapy-induced alterations in m6A modifications and METTL3 expression in HR+HER2- subtype BC, providing a theoretical basis for METTL3 as a new predictor of chemotherapy response and target for drug therapy.

Abbreviations

breast cancer (BC)
hormone receptors (HRs)
human epidermal growth factor receptor 2 (HER2)
HR positive and HER2 negative (HR+HER2-)
aromatase inhibitors (AIs)
progression-free survival (PFS)
pathological complete response (pCR)
disease-free survival (DFS)
overall survival (OS)
N6-methyladenosine (m6A)
messenger RNA (mRNA)
methyltransferase-like 3 (METTL3)
methyltransferase-like 14 (METTL14)
Wilms tumour 1 associated protein (WTAP)
fat mass and obesity-associated (FTO)
AlkB homolog 5 (ALKBH5)
BC stem cells (BCSCs)
triple-negative BC (TNBC)
doxorubicin (DOX)
cyclin-dependent inhibitor kinase 1A (CDKN1A)
epithelial- mesenchymal transition (EMT)
short-hairpin RNA (shRNA)
methylated RNA immunoprecipitation (MeRIP)
Gene ontology (GO)
Kyoto Encyclopedia of Genes and Genomes (KEGG)
differentially expressed genes (DEGs)

paclitaxel (PTX)

cisplatin (DDP)

**Declarations**

**Ethics approval and consent to participate**

The research protocol was reviewed and approved by the Ethical Committee of Second Xiangya Hospital of Central South University.

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets analysed during the current study are available in the Gene Expression Omnibus repository. ([https://www.ncbi.nlm.nih.gov/geo/](https://www.ncbi.nlm.nih.gov/geo/))

**Competing interests**

The authors declare that they have no competing interests

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**Authors' contributions**

Conceived and designed the study: DJOY and WJY. Development of methodology: TH, MDF and YTL. Acquisition of data: LYZ and QTC. Analysis and interpretation of data: DJOY, TH, HYH and YW. Writing of the manuscript: DJOY and TH. Administrative, technical, or material support: YC, MRZ, QYZ and WJY. All authors read and approved the final manuscript.

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Figures
Figure 1

METTL3 is silenced in HR+HER2- BC samples and cell lines. (A). The expression of m6A-related genes in the GSE87455 HR+/HER2- BC cohort. (B) (C). The mRNA and protein levels of METTL3 in paired HR+/HER2- BC tissues obtained before and after chemotherapy treatment. (D). The protein level of METTL3 in MCF-7 and MCF-7/ADR cell lines. (E) (F). The mRNA and protein levels of METTL3 in MCF-7 and T47D cell lines.
and T47D cells treated with DOX for 24 h and their corresponding control cells. (G) (H). Upregulation of METTL3 expression was significantly associated with longer OS and PFS in HR+/HER2- BC patients.

Figure 2

METTL3 is responsible for chemotherapy-induced m6A modifications, and METTL3 depletion enhances HR+/HER2- BC cell growth. (A). m6A levels in patient samples collected after chemotherapy compared with their corresponding controls. (B) (C) (D). m6A levels in MCF-7 cells treated with DOX, PTX or DDP for
24 h compared with those in the control cells. (E). The protein and mRNA levels of METTL3 in MCF-7 cells with knockdown or overexpression of METTL3 were measured by western blotting and qPCR, respectively. (F). The global m6A modification levels in METTL3-knockdown and control MCF-7 cells were determined by colorimetric analysis. (G). Knockdown of METTL3 improved the proliferation ability of MCF-7 cells in the presence or absence of DOX for 24 h. (H). Overexpression of METTL3 impaired the proliferation ability of MCF-7 cells in the presence or absence of DOX for 24 h. (I). Knockout of METTL3 improved the colony formation ability of MCF-7 cells in the presence or absence of DOX for 24 h (left panel). Quantification of the colony formation assay results (right panel). (J). Overexpression of METTL3 impaired the colony formation ability of MCF-7 cells in the presence or absence of DOX for 24 h (left panel). Quantification of the colony formation assay results (right panel). (K) (L). Cell cycle distribution of MCF-7 cells with knockdown or overexpression of METTL3 was analysed by flow cytometry. Ap, apoptosis phase, G1, DNA pre-synthesis phase, S, DNA synthesis phase, G2, DNA post-synthesis phase.
Figure 3

Inhibition of METTL3 drives BC cell metastasis in vitro and in vivo. (A). Knockout or overexpression of METTL3 affects MCF-7 cell migration in vitro as indicated by the wound healing assay. (B). Overexpression of METTL3 promotes MCF-7 cell apoptosis. (C). Overexpression of METTL3 effectively inhibited HR+/HER2- BC subcutaneous tumour growth in nude mice (n=8). (D) (E). The tumour volume
and weight were measured in the NC and METTL3 groups. (F). Tumours were excised and processed for immunohistochemical staining for Ki-67.

Figure 4

Identification of potential targets of the METTL3-mediated m6A modification in BC cells. (A) Venn diagram showing 498 overlapping genes with differential expression between the two cell lines. (B) (C). GO and KEGG analysis of the overlapping genes. (D) (E). The protein and RNA levels of METTL3 and
CDKN1A in MCF-7 cells with METTL3 knockdown were measured by western blot and qRT-PCR, respectively. (F). MeRIP with an anti-m6A antibody was performed in MCF-7 cells. The m6A modification on CDKN1A mRNA was reduced upon METTL3 knockdown. (G). E-cadherin, N-cadherin, Vimentin and GAPDH protein levels were examined by western blot in DOX-treated and control HR+/HER2- BC after 24 h. (H). Cell morphologic changes after downregulation of METTL3. (I). E-cadherin, N-cadherin, Vimentin, METTL3 and GAPDH protein levels were examined by western blot in HR+/HER2- BC cells transfected with lentiviruses carrying METTL3 and/or sh-HuR. (J). E-cadherin, Vimentin, METTL3 and GAPDH protein levels in the NC and METTL3 groups were examined by western blot.

**Figure 5**

Doxorubicin influences AKT activation and promotes EMT progression in HR+/HER2- BC cells. (A) (B). p-AKT, AKT and GAPDH protein levels were examined by western blot in DOX-treated and control HR+/HER2- BC cells after 24 h. (C). p-AKT, AKT, METTL3 and GAPDH protein levels were examined by western blot in HR+/HER2- BC cells transfected with lentiviruses carrying METTL3 and/or sh-HuR. (D). p-AKT, AKT, METTL3 and GAPDH protein levels in the NC, NC-DOX, METTL3 and METTL3-DOX groups were examined by western blot. (E). E-cadherin, N-cadherin, p-AKT, AKT, METTL3 and GAPDH protein levels in the shNC, shNC-LY294002, shMETTL3 and shMETTL3-LY294002 groups were examined by western blot. (F). p-AKT, AKT, METTL3, CDKN1A and GAPDH protein levels in the NC, shMETTL3 and shMETTL3-CDKN1A groups were examined by western blot.
Figure 6

METTL3 induces HR+/HER2- BC apoptosis via the m6A-Bax/caspase 3/8/9 pathway. (A) (B). METTL3 knockdown or overexpression influenced the expression levels of caspase proteins as indicated by the western blot assay. (C) (D). BAX expression was depleted upon METTL3 knockdown but enhanced by METTL3 overexpression. (E). MeRIP with an anti-m6A antibody was performed in MCF-7 cells. The m6A
modification on BAX mRNA was depleted upon METTL3 knockdown. (F). Schematic summarizing the role of METTL3 in modulating HR+HER2- BC progression.

Supplementary Files

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