The m6A Methyltransferase METTL3 Promotes Cisplatin Resistance and Invasion in Testicular Seminoma via BCL2

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Abstract

Background: Methyltransferase-like 3 (METTL3) involves in promoting tumor progression through tumor-related genes N6-methyladenosine (m6A) modification. Our previous study found that METTL3 plays an important role in seminoma chemosensitivity. BCL-2 is a key cause of cisplatin resistance in many tumors. Therefore, we want to explore whether METTL3 affects cisplatin resistance of seminoma by regulating BCL-2.

Methods: In this study, we downregulated and overexpressed METTL3 in TCam-2 cisplatin resistant cells (TCam-2/CDDP). Then, m6A RNA methylation quantification of BCL-2 and cell viability assay, cell apoptosis analysis and cell invasion assay were investigated under the condition of with or without cis-dichlorodiammine platinum (CDDP) treatment.

Results: Consistent with our previous results, METTL3 significantly affects the chemosensitivity of TCam-2/CDDP. After METTL3 downregulated, the proliferation and anti-apoptosis ability of TCam-2/CDDP cells significantly weakened. Correspondingly, overexpression of METTL3 could promote the chemoresistance. However, the phenotype could be partly reversed by decreasing the expression of BCL-2. Moreover, we found that the m6A modification of BCL-2 is more abundant in cisplatin-resistant strains. Knockdown and overexpression of METTL3 significantly affected the m6A modification and the protein level of BCL-2 in TCam-2/CDDP. Finally, we found that METTL3 also promoted the invasion ability of TCam-2/CDDP cells via BCL2.

Conclusion: This study revealed that METTL3 promotes anti-apoptosis and invasion of TCam-2/CDDP through BCL-2. And it indicated that METTL3 and BCL-2 may be an effective treatment target for CDDP-resistance seminoma.

Introduction

Testicular germ cell tumors (TGCTs) are the most commonly diagnosed solid malignancies in young men, and its incidence has been increasing worldwide in recent years [1]. The most common type is the testicular seminoma [2–4]. Furthermore, platinum-based chemotherapy is one of the essential comprehensive adjuvant therapies for TGCTs. However, some patients still cannot achieve remission after receiving platinum-based chemotherapy, which leads to treatment failure and eventually die [5–6]. Therefore, it is very important to explore the mechanism of chemoresistance and find targets for chemoresistant patients.

M6A refers to the methylation of nitrogen at position six of adenine and the most abundant modification in eukaryotic messenger RNAs (mRNAs) [7]. METTL3, an RNA methyltransferase, can promote translation, oncoprotein expression, cell proliferation, survival, and invasion [8]. It has been reported that METTL3 can encourage the translation of target mRNAs in specific cell types and play an essential role in the development of tumors such as bladder cancer, liver cancer, lung cancer, breast cancer and chronic leukemia [7–11]. M6A has a critical regulatory role in tumor stem cells and drug resistance [1],[12]. Our

previous study found that METTL3 mediates cisplatin resistance through autophagy and modified TFAP2C [13–14] Platinum resistance can also result from defects in initiation of apoptosis, yet the classical anti-apoptosis gene BCL2 have not been proven to be determinants for chemoresistance in germ cell tumors [15–17]. Therefore, this study intends to explore the role of METTL3 in promoting the resistance of TCam-2 cells via classical anti-apoptosis gene BCL2.

Materials And Methods

Statement

The Institutional Research Ethics Committee of The Fifth Affiliated Hospital of Sun Yat-sen University approved the study. All experiments adhered to the principles set forth in the Declaration of Helsinki.

Cell culture and Reagents

The tumor cell lines TCam-2 cells were kindly gifted from Dr. Riko Kitazawa (Department of Diagnostic Pathology, Ehime University Hospital, Matsuyama, Japan). Cells were maintained in complete medium (Roswell Park Memorial Institute Medium-1640 with 10% fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin) in a humidified 5% CO₂, 37 °C incubator. In our previous study, we established seminoma TCam-2/CDDP cell lines [14]. Cisplatin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies used in this study were METTL3 (Omnimabs, OM284614), BCL2 (Santa Cruz, sc-7382), BCLAF1 (Omnimabs, OM272372), MCL1 (Omnimabs, OM262629), GAPDH (Abcam, ab125247), Caspase-3 (Abcam, ab32351), and Cytc (Abcam, ab133504).

M6A RNA Methylation Quantification and MeRIP-qPCR

M6A RNA Methylation Quantification was performed as previously described [4-5]. Briefly, total RNA is bound to strip wells using an RNA high binding solution. N6-methyladenosine (m6A) is detected using a specific capture N6-methyladenosine antibody and detection antibody. The detected signal is enhanced and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer at a wavelength of 450 nm. The amount of m6A is proportional to the OD intensity measured. EZ-Magna RIP kit (Millipore) was used to evaluate the modification level of m6A in the specific gene according to the manufacturer's instructions. The enriched RNA-antibody complex was digested with protease to obtain m6A modified RNA. Then qRT-PCR was used to quantify the enriched RNA. Overall, the RNA containing m6A methylated was coated with m6A antibody and immunoprecipitated on magnetic beads A/G.

Western blotting

Cells were lysed in lysis buffer containing protease inhibitors for 30 minutes. The protein concentration of lysates was determined by the bicinchoninic acid assay (Beyotime Biotechnology). An equivalent amount of protein (30µg) of each sample was separated by 10% SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked with 5% non-fat dry milk in 0.2% Tween-20 in Tris-buffered saline for one h at room temperature and then probed with primary antibodies. After incubated in the secondary antibody, immunoreactivity was detected by the enhanced chemiluminescence method (Affinity biosciences, Cincinnati, OH, USA).

Cell viability assay

The cytotoxic effect of CDDP on TCam-2/CDDP cells was measured by cell count kit 8 (CCK8) assay. TCam-2 cells were seeded into 96-well plates (Corning Incorporated, Corning, NY, USA) at a density of 1×10^4 cells/well and cultured for 24 hours. Then, cells were cotreated with different concentrations of cisplatin in medium for 0, 24, 48, 72 hours. Thereafter, 10 µL of CCK-8 (Vazyme Biotech, Nanjing, China) solution was added to each well and mixtures were incubated at 37°C for additional 1 hour. The optical density (OD) at 450 nm was read with a microplate reader (Bio-Tek, Winooski, VT, USA).

Cell apoptosis analysis

Apoptosis was measured by Annexin V-FITC (fluorescein isothiocyanate)/propidium iodide (PI) staining. Briefly, after treatment and incubation for 72 h, cells were collected, washed with PBS, and labeled with Annexin V and propidium iodide in the dark using an Annexin V-FITC apoptosis detection kit (Vazyme Biotech, Nanjing, China). Cell apoptosis was subsequently analyzed by a flow cytometry (Beckman Coulter, Inc. Brea, CA, USA)

Cell invasion assay

A cell invasion assay was performed using Transwell chambers(Corning, NY, USA) with 8-µm pore inserts. Cell suspensions were seeded in the upper chamber, and 500 µL of Dulbecco's Modified Eagle's medium containing fetal bovine serum was added to the lower chamber. The noninvading cells were removed with a cotton-tipped swab after 24 hours of incubation, and the invading cells on the bottom surface of the membrane were stained with 0.1% crystal violet. The invading cells were quantified by counting ten random fields at $\times 200$ magnification.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from cells using the TRIzol reagent of Vazyme Biotech (Nanjing, People's Republic of China) following the manufacturer's protocol. The PCR primer sequences were as follows: METTL3 forward primer 5'-TTGTCTCCAACCTTCCGTAGT-3', reverse primer 5'-

CCAGATCAGAGAGGTGGTGTAG-3' BCL2 forward primer 5'-GGTGGGGTCATGTGTGTGG-3', reverse primer 5'-CGGTTCAAGTACTCAGTCATCC-3' MCL1 forward primer 5'-TGCTTCGGAACTGGACATCA-3', reverse primer 5'-TAGCCACAAAGGCACCAAAAG-3'; BCLAF1 forward primer 5'- ATGAGACGACCTTATGGGTACA-3', reverse primer 5'- AGAGTGCCTTCTATTCCAGACAG-3'; GAPDH forward primer 5'- GGAGCGAGATCCCTCCAAAAT-3', reverse primer 5'- GGCTGTTGTCATACTTCTCATGG -3'.

Cell transfection

SiRNAs for METTL3 and BCL2 were designed. The siRNA sequences were as follows: siRNA-METTL3, sense: 5'-GCAAGUAUGUUCACUAUGATT-3', antisense: 5'-UCAUAGUGAACAUACUUGCAG-3'; siRNA-BCL2, sense: 5'- GCATGCGGCCTCTGTTTGATT-3', antisense: 5'-UCAUAGUGAACAUACUUGCAG-3. Both plasmids and siRNA were transfected with LipofectamineTM 3000 (ThermoFisher, MA, USA) reagent and transfected when the cells met at 70% confluent. The plasmid overexpressing METTL3 has constructed previously [14].

Data processing

Data were analyzed with Prism 7 (GraphPad Software) and presented as mean $\pm$ standard deviation (SD). The student's t test was used to analyze the statistical significance of the difference between the two groups. Values are presented as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Results

METTL3 can enhance the anti-apoptosis ability of TCam-2/CDDP cells

It has been proved that m6A modification involved in the occurrence and development of tumor drug resistance [17]. We have detected m6A modification of total RNA in TCam-2 cell line and TCam-2/CDDP cell line, and the results showed that the m6A modification in total RNA of TCam-2/CDDP cells was significantly higher than that of TCam-2 cells [13]. Then, siRNA or plasmid was used for knockdown (si-METTL3) or overexpression (OE-METTL3) of METTL3 in TCam-2/CDDP cells, respectively.

The transfection efficiency was validated by qRT-PCR (Figure 1A). Si-METTL3 group and OE-METTL3 group were treated with cisplatin at IC₂₅ concentration [18]. The toxicity of cisplatin to these two groups was detected by CCK8 assay. The results indicated that knockdown of METTL3 significantly inhibited cell viability, while upregulation of METTL3 could accelerate the viability slightly (Figure 1B). As shown in figure 1C and 1D, apoptosis rate was significantly higher in Si-METTL3 group, and lower in OE-METTL3 group compared to NC group. Meanwhile, apoptosis-related proteins Caspase3 and Cyt c expression were

much higher than NC group after METTL3 downregulated (Figure 1E). The aforementioned results reveal that METTL3 increases the chemotherapy resistance of TCam-2 cells through anti-apoptosis.

(A) Relative mRNA of METTL3 in NC, OE-METTL3 and Si-METTL3 groups (B) Cell viability analysis of TCam-2/CDDP treated with CDDP at different time. (C) Cells in different groups were treated with cisplatin for 72 h, followed by staining with apoptosis detection kit and analyzed by flow cytometry. (D) Quantitative summary of the apoptosis rate in the different treatment groups. (E) Western blot analysis of caspase3 and cytc in the different groups. GAPDH served as the internal control.

METTL3 involves in regulation of BCL2 in TCam-2/CDDP cells

Researchers have suggested that m6A modification can induce the occurrence of anti-apoptosis in cancers by regulating the expression of classical anti-apoptosis genes [19–20]. In our previous study, we have proved that METTL3 confers resistance to cisplatin in TCam-2 cells [13]. Thus, we speculated that METTL3 could regulate the anti-apoptosis related genes through m6A modification and thus induce chemoresistance. We used m6A-IP-qPCR to evaluate m6A modification of apoptosis-related gene BCL2, BCLAF1 and MCL1. As shown in Figure 2A, the m6A modification of BCL-2 was higher in drug-resistant lines. And the proteins expression of BCL-2 decreased significantly after METTL3 knockdown. The aforementioned results revealed that the higher expression of BCL-2 in TCam-2/CDDP was regulated by METTL3.

METTL3 can promote the anti-apoptosis ability of TCam-2/CDDP cells via BCL2

To elucidate the mechanism underlying the anti-apoptosis ability triggered by METTL3, we focused on the BCL2 pathway. As shown in figure 3A and 3B, the BCL2 related m6A modification, and related mRNA expression significantly decreased after METTL3 knockdown. Furthermore, upregulation of METTL3 strengthened the anti-apoptosis ability of TCam-2/CDDP cells. However, the phenotype could be reversed by downregulation of BCL-2(Figure 3D E). These results illustrate that m6A modification of BCL-2 by METTL3 promotes the chemoresistance of TCam-2/CDDP cells.

METTL3 can also enhance the invasion of TCam-2/CDDP cells via BCL2

Recent studies have shown that drug-resistant cells have a stronger ability to invade [21–22], and BCL2 has the ability to promote invasion and metastasis [23]. Our results shown that the invasion ability of TCam-2/CDDP cells was much higher after METTL3 overexpressed, and a contrary result was observed when METTL3 knocked down. Meanwhile, the promotion of cell invasion induced by METTL3 upregulation was significantly decreased after BCL2 knockdown (Figure 4). Thus, the result illustrated that METTL3 can enhance the invasion ability of TCam-2 cells via BCL2.

Discussion

m6A is the most abundant internal modification in eukaryotic mRNA and plays a vital role in normal biological processes [24–25], includes self-renewal, differentiation of embryonic stem cells, and DNA damage etc [26–39]. Furthermore, emerging evidence has indicated that m6A RNA methylation plays an important role in tumor initiation and progression [40]. Meanwhile, some researchers found mRNA expression levels of VIRMA, YTHDF3, METTL4, ALKBH5 and YTHDC1 were significantly higher in seminoma compared to non- seminoma, which indicated m6A related markers are related to the monitoring and identification of TGCTs (PMID: **30428628**). Other researchers' study revealed that the expression of METTL3 increased in TGCTs' cell lines and tissues (PMID: **30903744**). METTL3, as m6A writers, involves in many biological processes of tumors as mentioned above. A recent study revealed that ARHG AP5-AS1 stabilized ARHGAP5 mRNA by recruiting METTL3 to stimulate m6A modification of ARHGAP5 mRNA and contribute to drug resistance, indicating that METTL3 was involved in chemotherapy resistance in gastrointestinal cancer [41]. Similarly, mechanistic studies have shown that METTL3 combines with MET and causes the PI3K/AKT signaling pathway to be manipulated, which affects the sensitivity of lung cancer cells to gefitinib [42].

Cisplatin-based chemotherapy plays an important role in the treatment of seminoma. Unfortunately, seminoma is also lethal when gaining resistance to therapy. However, the progress of seminoma and the mechanism of drug resistance are still unclear. More researches on the mechanisms of drug resistance can provide a new direction for clinical treatment. In our previous study, we found that METTL3 was involved in chemoresistance of TCam-2 cells, and promoted CDDP-resistance by m6A modification of ATG5 and TFAP2C. Many researches have revealed that the platinum- resistance often result from defects in initiation of apoptosis, and many studies determine that BCL2, BCLAF1 and MCL1 are important oncogenes involved in cancer by inhibiting apoptosis [15–17]. Therefore, we explored whether METTL3 affected apoptosis to cause drug resistance in seminoma. Likely, we analyzed the apoptosis ratio of TCam-2/CDDP cells cisplatin after CDDP treatment, and found that downregulation of METTL3 obviously alleviated the anti-apoptotic property of cells. These results verify the hypothesis that METTL3 promotes drug resistance of seminoma through decreasing apoptosis.

To further explore the anti-apoptosis mechanism of METTL3, we selected candidate genes via literature retrieval and online database prediction. Through western blot and MeRIP-qPCR, we finally chose BCL-2 as our target. After knockdown of METTL3 in TCam-2/CDDP cells, the mRNA level of BCL-2 was evidently decreased, as well as the m6A abundance. This result revealed that METTL3 could aggrandize the mRNA level of BCL-2 through m6A modification. our results also shown that downregulation of BCL-2 could reverse the effect of METTL3 on anti-apoptosis. The aforementioned results verified METTL3 can promote the anti-apoptosis ability of TCam-2/CDDP cells via m6A modification of BCL2.

However, Metastasis is the worst prognosis predictor in the clinical course of cancer development. Features of metastatic cancer cells include migratory ability, low degree of differentiation, self-renewal and proliferation potentials, as well as resistance to therapies (PMID: 31813120). Although BCL-2 family

proteins were originally identified as key regulators of apoptosis, an impressive body of evidence has shown that pro-survival members of the BCL-2 family can also promote cell migration, invasion, and cancer metastasis (PMID: **26621844**). Our study validated that overexpression of METTL3 enhanced the migration ability of TCam-2/CDDP cells, while siRNA BCL-2 reversed the potential promotion. Therefore, METTL3/BCL-2 participates the migration of TCam-2/CDDP cells.

In summary, we verified the mechanism of METTL3 promoting drug resistance and migration of seminoma through m6A modification of BCL-2 in vitro. However, some limitations should be acknowledged in this study. It remains unclear how METTL3 regulates the mRNA level of BCL-2, and how BCL-2 regulates the apoptosis and migration in seminoma. This study also lacks experiment in vivo to evaluate the conclusions. Furthermore, drug resistance in TGCTs occurs mostly in non-seminoma, the function of METTL3 in non-seminoma needs further study.

Conclusions

In summary, our study demonstrates the critical role of METTL3 in the process of seminoma resistance, which is characterized by promoting the expression of BCL-2 related proteins or RNA stability to promote the anti-apoptotic ability of TCam-2 cells, and speculated that that METL3 and BCL-2 may be therapeutic targets for drug-resistant seminoma.

Abbreviations

METTL3

Methyltransferase-like 3

m6A:N6-methyladenosine

TCam-2/CDDP

TCam-2 cisplatin resistant cells

CDDP

cis-dichlorodiammine platinum

TGCTs

Testicular germ cell tumors

mRNAs

messenger RNAs.

Declarations

Acknowledgements

The tumor cell lines TCam-2 cells were kindly gifted from Dr. Riko Kitazawa (Department of Diagnostic Pathology, Ehime University Hospital, Matsuyama, Japan)

Author contributions

YXT and YLX designed the study; JXP, WFL, YHY and HFC performed the experiments, analyzed data and prepared the figures; DYP, YHY and QC contributed to drafting the manuscript. All authors read and approved the final manuscript.

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Data availability statement

The data used in the current study are available from the corresponding author on reasonable request.

Availability of data and materials

The data supporting the conclusions of this study are included within the article.

Ethics approval and consent to participate

The present study was approved by the Institutional Research Ethics Committee of The Fifth Affiliated Hospital of Sun Yat-sen University (Approval No. L020-1). Consent for publication Not applicable.

Competing interests

The authors declare that there were no conflicts of interest.

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Figures

Figure 1

METTL3 enhances the anti-apoptosis ability of TCam-2/CDDP cells (A) Relative mRNA of METTL3 in NC, OE-METTL3 and Si-METTL3 groups (B) Cell viability analysis of TCam-2/CDDP treated with CDDP at different time. (C) Cells in different groups were treated with cisplatin for 72 h, followed by staining with apoptosis detection kit and analyzed by flow cytometry. (D) Quantitative summary of the apoptosis rate in the different treatment groups. (E) Western blot analysis of caspase3 and cytc in the different groups. GAPDH served as the internal control.

Figure 2

METTL3 involves in the m6A modification of BCL2 in TCam-2/CDDP cells. (A) M6A-IP-qPCR tested the m6A modification of BCL2, MCL1 and BCLAF1. (B, C) Western blot analysis of METTL3, BCL2, BCLAF1 and MCL1 in the different treatment groups. GAPDH served as the internal control.

Figure 3

METTL3 cells enhance the anti-apoptosis ability of TCam-2/CDDP via BCL2. (A, B) m6A modification level of BCL-2 and mRNA expression in total RNA of NC, OE-METTL3 and Si-METTL3 groups. (C) BCL2 mRNA expression in NC and Si- BCL2 groups. (D) Left: Cells in different groups were treated with cisplatin for 72 h, followed by staining with apoptosis analysis kit and then analyzed by flow cytometry. Right:

Quantitative summary of the apoptosis rate in different groups. (E) Western blot analysis of caspase3 and cytc in different groups. GAPDH served as the internal control.

Figure 4

METTL3 enhanced the invasion ability of TCam-2 cells via BCL2. A: Representative images of cells invasion in different groups. B: Quantitative summary of cell numbers in different groups.

Figure 5

Caption not included with this version.