



Original Article

METTL3-mediated m6A modification enhances ZDHHC16 expression in non-small-cell lung cancer patients, attenuating ferroptosis by suppressing CREB ubiquitination

Zeyu Liu¹, Chuanqing Jing¹, Wei Zhang^{2*}¹ The First Clinical College of Shandong University of Traditional Chinese Medicine, Jinan 250001, China² Department of Respiratory and Critical Care Medicine, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan 250001, China

Article Info

Abstract



Article history:

Received: April 20, 2023

Accepted: January 12, 2024

Published: February 29, 2024

Use your device to scan and read the article online



At present, the early diagnosis and treatment of non-small cell lung cancer (NSCLC) is still an urgent problem to be solved worldwide, including in China. The present work investigated the possible protective effect of ZDHHC16 in cell proliferation and metastasis of NSCLC and explored its possible mechanisms. ZDHHC16 expression level in patients with Non-Small-Cell Lung Cancer was up-regulation. ZDHHC16 gene is stabilized by m6A methylation. ZDHHC16 gene reduced ferroptosis of NSCLC by the rehabilitation of the mitochondrial structure. ZDHHC16 promoted CREB expression through the inhibition of CREB Ubiquitination. Confocal microscopy showed that ZDHHC16 reduced the CREB expression of NSCLC. ZDHHC16 up-regulation reduced CREB Ubiquitination, and down-regulation of ZDHHC16 promoted CREB Ubiquitination of NSCLC. CREB Agonists reduced the effects of ZDHHC16 on ferroptosis, not affecting the Warburg effect of NSCLC. CREB inhibitor reduced the effects of si-ZDHHC16 on ferroptosis, not affecting the Warburg effect of NSCLC. METTL3-mediated m6A modification increases ZDHHC16 stability. Our study revealed that the m6A-forming enzyme METTL3 upregulates ZDHHC16 expression in NSCLC patients, leading to the reduction of ferroptosis by inhibiting CREB ubiquitination.

Keywords: Non-small cell lung cancer, ZDHHC16, CREB, Ubiquitination, m6A

1. Introduction

Lung cancer, the most common form of cancer, is the leading cause of cancer-related death [1]. Among them, NSCLC is the most common type of lung cancer, accounting for about 85% of the total [2]. NSCLC is a malignant tumor that seriously endangers health, with high morbidity and mortality. Despite the rapid development of medical technology, the overall efficacy of NSCLC is still unsatisfactory [3]. Therefore, in-depth research on the molecular biological characteristics and pathogenesis of NSCLC is of great significance for new therapeutic targets [3].

Ferroptosis is a kind of programmed cell death mode different from apoptosis, necrosis and autophagy, which was found in recent years [4]. Its main mechanism is to catalyze the lipid peroxidation of highly expressed unsaturated fat acids in cells under the action of divalent iron or lipoxygenase, thus inducing cell death [5]. Ferroptosis is involved in the occurrence of various diseases, such as tumors, rheumatoid arthritis, neurodegenerative diseases, ischemia-reperfusion, and heart-related diseases [6]. Lung

cancer is the most lethal malignant tumor in the world, and its treatment strategies are constantly being updated [7]. In recent years, an increasing number of research results have shown a close relationship between ferroptosis and NSCLC [8]. Some molecules that play important regulatory roles in the occurrence, development, and treatment of NSCLC (such as KRAS, TP53, EGFR, etc.) also play a role in the occurrence of ferroptosis [9]. In addition, ferroptosis is closely related to chemotherapy, radiation therapy, and immunotherapy in NSCLC. Some preclinical studies have confirmed that ferroptosis can serve as a "catalyst", and the combination of the above treatment methods can significantly enhance the therapeutic effect [10, 11].

During the aerobic metabolism process of cells, a series of reactive oxygen species (ROS) are produced [12]. Excessive levels of ROS can cause damage to the cell and gene structure [12]. CREB can alleviate the toxic damage of ROS to cells by acting on the H2O2 enzyme, thereby maintaining mitochondrial activity under oxidative stress and promoting cell survival [13]. CREB silencing can lead

* Corresponding author.

E-mail address: huxizhijia@126.com (W. Zhang).Doi: <http://dx.doi.org/10.14715/cmb/2024.70.2.5>

to an increase in cell apoptosis after oxidative stress [14]. The CREB treated with H₂O₂ can promote DNA repair [14]. They used cDNA microarray analysis to show that CREB can regulate the DNA repair involved in post-oxidative damage and the expression of multiple genes related to cell survival, to mediate cell proliferation and induce the formation of cell anti-apoptosis pattern formation [14].

In NSCLC, ZDHHC16 was found to be a dominant enzyme that relies on claudin 3 (CLDN3) for palmitoylation. Through palmitoylation, ZDHHC16 stabilizes the CLDN3 protein and promotes the occurrence and development of cancer [15, 16]. Recent studies have found that ZDHHC16 regulates the progression and metastasis of colorectal cancer, suggesting that interfering with ZDHHC16 may be a feasible method for treating advanced metastatic colorectal cancer [17, 18].

It is reported that the expression of m6A methylation modification has been found to increase or decrease in various cancers, and its role in cancer is gradually being explored [19]. Currently, the study of m6A methylation during the growth and invasion of various tumors is one of the hotspots in tumor biology research [20]. Especially in the growth and invasion process of NSCLC, m6A methylation plays a crucial role [21]. Abnormal expression of m6A-related proteins is associated with malignant proliferation, migration, invasion, metastasis, and drug resistance in NSCLC [22]. The present work investigated the possible protective effect of ZDHHC16 in cell proliferation and metastasis of NSCLC and explored its possible mechanisms.

2. Materials and methods

2.1. Clinical samples

Patients with NSCLC (n = 24) were obtained from Affiliated Hospital of Shandong University of Traditional Chinese Medicine from August 2013 to August 2014. All the samples were obtained following patient consent and approval by the Ethics Committee of Affiliated Hospital of Shandong University of Traditional Chinese Medicine. Tissue samples were collected and saved at -80°C. Overall survival (OS) and disease-free survival (DFS) were executed and followed for 5 years.

2.2. Cell culture and transfection

Human lung fibroblasts CCD-19Lu, NSCLC (A549, NCL-H129, NCL-H3122 and NCL-H466 cell), HEK293T cells were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS, Gibco, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO₂ at 37°C. Plasmids were transfected into NSCLC cells using Lipofectamine 2000.

2.3. Quantitative PCR

The total RNA was extracted from serum and cell samples using a TRIZOL reagent (Life Technologies Inc.). qRT-PCR assays were performed using Light Cycler® 480 SYBR Mix (Roche, Germany) using LightCycler® 480 real-time PCR system. The expression levels of mRNA were normalized to the GAPDH expression using the 2- $\Delta\Delta$ ct method.

2.4. Proliferation assay and EDU staining

After 48 h of transfection, a total of approximately

2×10³ cells/well was seeded in a 96-well plate. After culturing at the indicated time (0, 6, 12, 24 and 48 days), the cellular proliferation was detected using CellTiter-GloR Luminescent Cell Viability Assay (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions. EdU (10 mM) was added to each well and cells were fixed with 4% formaldehyde for 30 min. After washing, EdU was detected with Click-iTR EdU Kit and images were visualized using a fluorescent microscope (Olympus).

Glucose consumption, lactate production and ATP level measurement, Extracellular Acidification Rate (ECAR) and oxygen consumption rate (OCR).

Glucose was determined by a glucose assay kit (Beyotime). Lactate levels were measured by Lactate Colorimetric/Fluorometric Assay Kit (Nanjing Jiancheng Bioengineering Research Institute Co., Ltd.). ATP level was determined using the ATP Determination Kit (Beyotime). ECAR and OCR were determined using the Seahorse XFe96 analyzer and OCR (Seahorse Bioscience, Agilent). The stable transfected cells (1×10⁴ cells/well) were seeded into 96-well XF cell culture microplates. After 24 h, the medium was respectively replaced by an XF base medium (pH 7.4) containing glucose (10 mM), glutamine (1 mM), 2-DG (50 mM) and oligomycin (1 μM). Finally, the ECAR was detected using an XFe96 analyzer (Seahorse Bioscience). For the OCR, cells were respectively treated with oligomycin (1 mM), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and antimycin A (2 mM) and rotenone. The data of ECAR and OCR was measured normalized to total protein content (mpH/min).

2.5. Western blot analysis

Total protein was extracted from cell samples using Radio-Immunoprecipitation Assay (RIPA) and PMSF reagent (Beyotime, Beijing, China). Protein lysates were separated based on their molecular weight on SDS/PAGE gels and transferred onto a Polyvinylidene Fluoride (PVDF, Millipore) membrane. The membranes were blocked with non-fat-milk (5%) for 2 h at room temperature and incubated with anti-KLF13 antibody, anti-PPAR γ antibody and anti- β -actin antibody at 4°C overnight. Then first antibodies were removed and the TBST wash membrane using TBST. Membranes were incubated with the secondary antibody for 2 hours at room temperature. The bound antibodies were detected using enhanced chemiluminescence (ECL) with β -actin used as a control.

2.6. Immunofluorescence

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature, and blocked with 5% BSA for 30 min at 37°C. Cells were treated with primary antibodies: anti-KLF13 antibody, and anti-PPAR γ antibody at 4°C overnight. Cells were then incubated with Cy3-conjugated goat anti-rabbit or goat anti-mouse IgG DyLight 488-conjugated secondary antibodies for 2 h at 37°C. Nuclei were stained with DAPI and cells were observed under a fluorescent illumination microscope (Olympus IX71, Tokyo, Japan).

2.7. Statistical analysis

Data were analyzed with GraphPad 8.0 Software (La Jolla, CA, USA) and reported as the mean \pm SD. The differences between groups were analyzed using Student's t-test or two-way ANOVA with repeated measures followed

by the Tukey post hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. ZDHHC16 expression level in a model of NSCLC

We first confirmed the expression levels of ZDHHC16 in a model of NSCLC. We collected patients with NSCLC ($n = 24$). There was an increase in ZDHHC16 mRNA expression in patients with NSCLC, compared with the normal group (Figure 1A). The expression of ZDHHC16 mRNA levels in NSCLC cell lines were up-regulated, compared with normal lung cell line (Figure 1B). Additionally, the DFS and OS of ZDHHC16 with lower expression in patients with NSCLC were higher than those of ZDHHC16 with higher expression (Figure 1C-1D). Taken together, it suggested that ZDHHC16 played a repair factor in the replantation of NSCLC.

3.2. ZDHHC16 gene is stabilized by m6A methylation

The study investigated the mechanism of ZDHHC16 on cell proliferation and progression of NSCLC. RNA m6A methyltransferase METTL3 and METTL14 levels were upregulated in patients with NSCLC (Figure 2A-2B). The high expression of METTL3 or METTL14 is positively correlated with the level of ZDHHC16 in NSCLC (Figure 2C-2D). ZDHHC16 is enriched in fraction immunoprecipitated by m6A anti-body (Figure 2E). METTL3 and METTL14 depleted and reduced the m6A methylation level of ZDHHC16 in NSCLC (Figure 2F). Si-METTL3 or si-METTL14 decrease ZDHHC16 gene expression levels (Figure 2G), suggesting that m6A methylation enhances the stability of ZDHHC16.

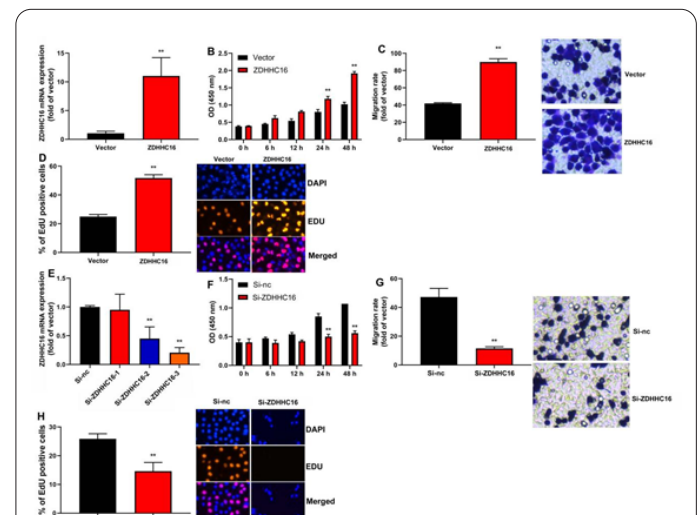
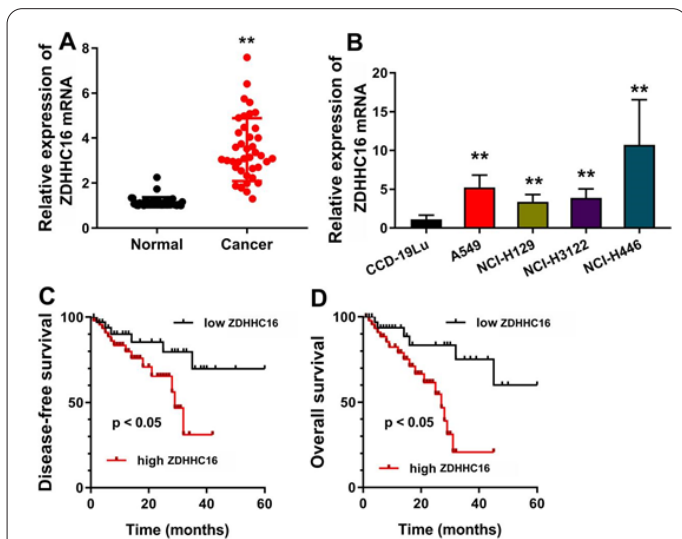
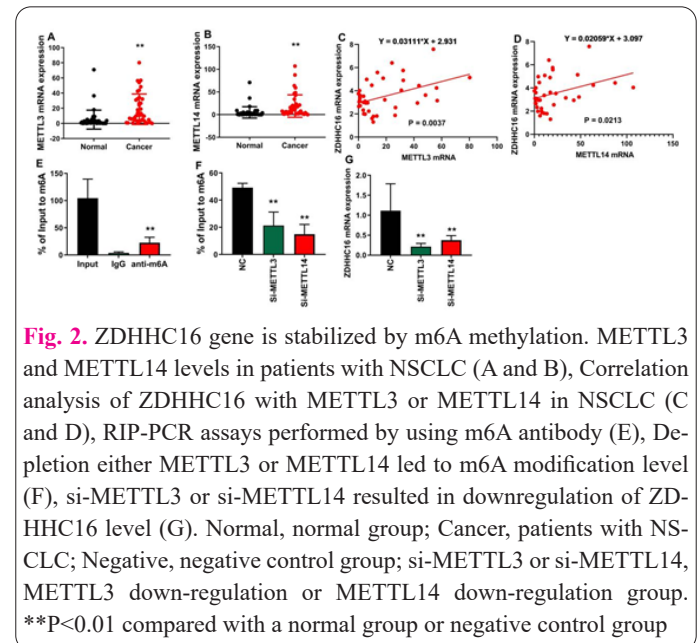
3.3. ZDHHC16 gene promoted cell Proliferation and Metastasis of NSCLC

The experiment determined the effects of ZDHHC16 in the cell Proliferation and Metastasis of NSCLC, we first measured cell proliferation and metastasis of NSCLC in vitro model. ZDHHC16 up-regulation increased the ex-

pression of ZDHHC16 mRNA level in NSCLC (Figure 3A). ZDHHC16 up-regulation promoted cell growth and increased the number of EDU cells and the migration rate of NSCLC (Figure 3B-3D). Si-ZDHHC16 reduced ZDHHC16 mRNA expression level in NSCLC (Figure 3E). Down-regulation of ZDHHC16 reduced cell growth and inhibited the number of EDU cells and migration rate of NSCLC (Figure 3G-3H). Taken together, our data suggest that ZDHHC16 promoted cell growth of NSCLC.

3.4. ZDHHC16 gene promoted Warburg effect of NSCLC

We further examined that the function of ZDHHC16 affected the Warburg effect of cervical carcinoma. Over-expression of ZDHHC16 promoted glucose consumption, lactate production and ATP quantity (Figure 4A-



4C). Down-regulation of ZDHHC16 reduced glucose consumption, lactate production and ATP quantity (Figure 4A-4C). Over-expression of ZDHHC16 promoted extracellular acidification rate (ECAR) and down-regulation of ZDHHC16 reduced ECAR in vitro model of cervical carcinoma (Figure 4D-4E). Over-expression of ZDHHC16 reduced the OCR relative level and down-regulation of ZDHHC16 promoted the OCR relative level in vitro model of cervical carcinoma (Figure 4F-4G). In general, data suggests that ZDHHC16 promoted the Warburg effect of NSCLC.

3.5. ZDHHC16 gene reduced ferroptosis of NSCLC by the rehabilitation of mitochondrial structure

This study examined the function of ZDHHC16 on ferroptosis of NSCLC. Over-expression of ZDHHC16 reduced LDH activity levels and PI cells, inhibited iron concentration, increased GPX4 protein expression, promoted MPT and JC-1 levels, and rehabilitated the mitochondrial structure of NSCLC (Figure 5A-5G). Down-regulation of ZDHHC16 increased LDH activity levels and PI cells, promoted iron concentration, decreased GPX4 protein expression, suppressed MPT and JC-1 levels, and reduced mitochondrial structure of NSCLC (Figure 5H-5N).

3.6. ZDHHC16 promoted CREB expression through the inhibition of CREB Ubiquitination

Next, the study further investigated the mechanism of ZDHHC16 on cell proliferation and progression of NSCLC. Over-expression of ZDHHC16 induced ZDHHC16 and p-CREB protein expressions of NSCLC (Figure 6A). Down-regulation of ZDHHC16 suppressed ZDHHC16 and p-CREB protein expressions of NSCLC (Figure 6B). Confocal microscopy showed that ZDHHC16 reduced the CREB expression of NSCLC (Figure 6C). Live ima-

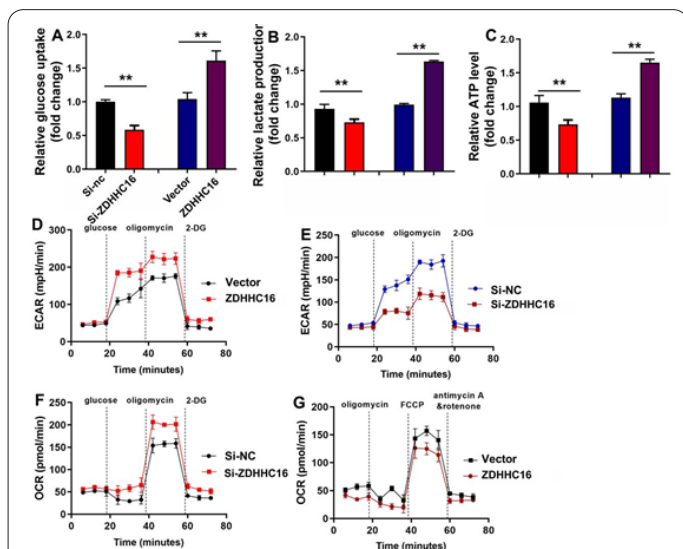


Fig. 4. ZDHHC16 gene promoted the Warburg effect of NSCLC. Glucose consumption analysis revealed glucose consumption (A), Lactate production analysis revealed lactate production (B), ATP quantity analysis revealed the ATP quantity (C), lactate-induced acidification of the medium surrounding cells (D and E), mitochondrial respiratory capacity was conducted using Seahorse XFp assay (F and G). Vector, negative control group; ZDHHC16, over-expression of ZDHHC16 group; Si-nc, si-negative control group; Si-ZDHHC16, down-regulation of ZDHHC16 group; ** $P < 0.01$ compared with negative control group or si-negative control group.

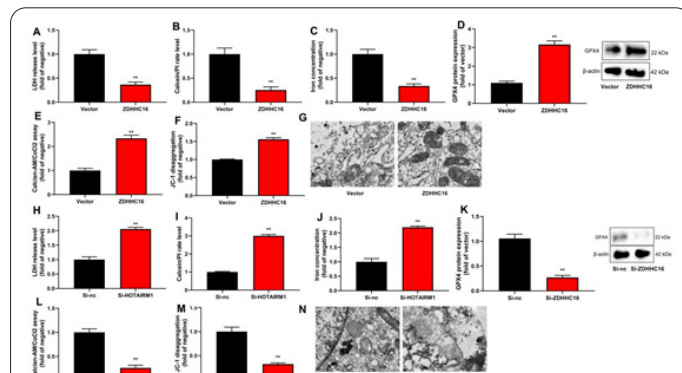


Fig. 5. ZDHHC16 gene reduced ferroptosis of NSCLC by the rehabilitation of mitochondrial structure. LDH (A), PI cells (B), iron concentration (C), GPX4 protein expression (D), MPT assay (E), JC-1 levels (F), mitochondrion (electron microscope, G) in vitro model of NSCLC by over-expression of ZDHHC16; LDH (H), PI cells (I), iron concentration (J), GPX4 protein expression (K), MPT assay (L), JC-1 levels (M), mitochondrion (electron microscope, N) in vitro model of NSCLC by down-regulation of ZDHHC16. Vector, negative control group; ZDHHC16, over-expression of ZDHHC16 group; Si-nc, si-negative control group; Si-ZDHHC16, down-regulation of ZDHHC16 group; ** $P < 0.01$ compared with negative control group or si-negative control group.

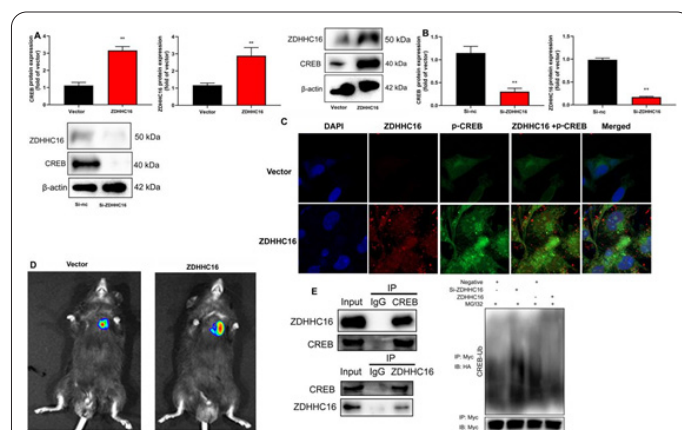


Fig. 6. ZDHHC16 promoted CREB expression through the inhibition of CREB Ubiquitination. ZDHHC16 and p-CREB protein expression (A and B), ZDHHC16 and CREB expression using confocal microscopy (C), p-CREB expression (vivo imaging, D), ZDHHC16 protein and p-CREB protein/CREB Ubiquitination (E). Vector, negative control group; ZDHHC16, over-expression of ZDHHC16 group; Si-nc, si-negative control group; Si-ZDHHC16, down-regulation of ZDHHC16 group; ** $P < 0.01$ compared with negative control group or si-negative control group.

ging display, ZDHHC16 increased CREB expression of NSCLC in mice model (Figure 6D). The endogenous proteins that showed robust interaction between ZDHHC16 protein and CREB protein were confirmed by IP (Figure 6E). ZDHHC16 up-regulation reduced CREB Ubiquitination and down-regulation of ZDHHC16 promoted CREB Ubiquitination of NSCLC (Figure 6E). Thus, our finding suggests ZDHHC16 induced CREB expression through the inhibition of CREB Ubiquitination.

3.7. CREB Agonists reduced the effects of ZDHHC16 on ferroptosis, not affect the Warburg effect of NSCLC

The study identified the role of CREB on ferroptosis and Warburg effect of NSCLC. CREB agonists (5 μM of compound AE-18) induced CREB and GPX4 protein

expression, promoted cell proliferation and metastasis, reduced ferroptosis and mitochondrial damage, and did not affect the Warburg effect of NSCLC by ZDHHC16 (Figure 7).

3.8. CREB inhibitor reduced the effects of si-ZDHHC16 on ferroptosis, not affecting the Warburg effect of NSCLC

Meanwhile, CREB inhibitor (0.5 μM CREB-IN-1 TFA) suppressed CREB and GPX4 protein expression, reduced cell proliferation and metastasis, promoted ferroptosis and mitochondrial damage, and did not affect the Warburg effect of NSCLC by ZDHHC16 (Figure 8). In general, data suggests that ZDHHC16 promoted cell proliferation and metastasis of NSCLC through the inhibition of ferroptosis by CREB.

3.9. METTL3-mediated m6A modification increases ZDHHC16 stability

We examined the molecular mechanism underlying of ZDHHC16 in NSCLC. ZDHHC16 has multiple suspicious methylation modification sites near the stop codon (Figure 9A). m6A antibody inhibited ZDHHC16 mRNA enrichment in NSCLC by METTL3 knockout, si-METTL3 also reduced the stability of ZDHHC16 mRNA in NSCLC (Figure 9B-9C). Up-regulation of METTL3 induced ZDHHC16 protein expression and silencing METTL3 inhibited ZDHHC16 protein expression in NSCLC (Figure 9D-9E). m6A sites in the 3'-untranslated region (UTR) of ZDHHC16 at sites 1, 2, 3 and 4 (Figure 9F). Si-METTL3 reduced luciferase activity level by wild-type (WT) of ZDHHC16, meanwhile, while the mutant (Mut) ZDHHC16 did not (figure 9G). The m6A enrichment of ZDHHC16 at sites 1, 2, 3 and 4 decreased METTL3 levels, and the m6A modification of ZDHHC16 mRNA by METTL3 (Figure 9H-9I). These results revealed that METTL3-mediated m6A modification increases ZDHHC16 stability.

4. Discussion

As we all know, lung cancer is the number one cancer in the world [23]. The 5-year survival rate for lung cancer

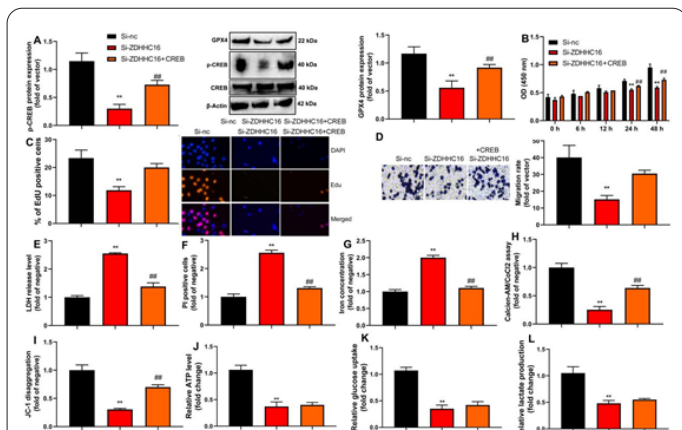


Fig. 7. CREB Agonists reduced the effects of ZDHHC16 on ferroptosis, not affected the Warburg effect of NSCLC. p-CREB/GPX4 protein expression (A), cell growth (B), EDU assay (C), migration rate (D), LDH (E), PI cells (F), iron concentration (G), MPT assay (H), JC-1 levels (I), ATP quantity (J), glucose consumption (K), lactate production (L). Si-nc, si-negative control group; Si-ZDHHC16, down-regulation of ZDHHC16 group; CREB, CREB Agonists; **P<0.01 compared with si-negative control group; ##P<0.01 compared with down-regulation of ZDHHC16 group.

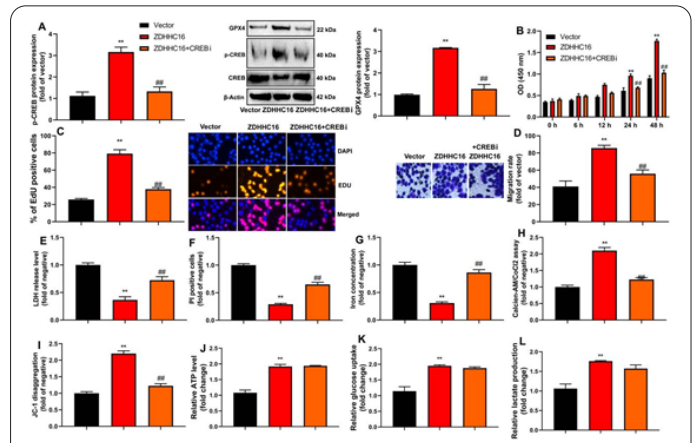


Fig. 8. CREB inhibitor reduced the effects of si-ZDHHC16 on ferroptosis, not affecting the Warburg effect of NSCLC. p-CREB/GPX4 protein expression (A), cell growth (B), EDU assay (C), migration rate (D), LDH (E), PI cells (F), iron concentration (G), MPT assay (H), JC-1 levels (I), ATP quantity (J), glucose consumption (K), lactate production (L). Vector, negative control group; ZDHHC16, over-expression of ZDHHC16 group; CREB i, CREB inhibitor; **P<0.01 compared with the negative control group; ##P<0.01 compared with over-expression of ZDHHC16 group.

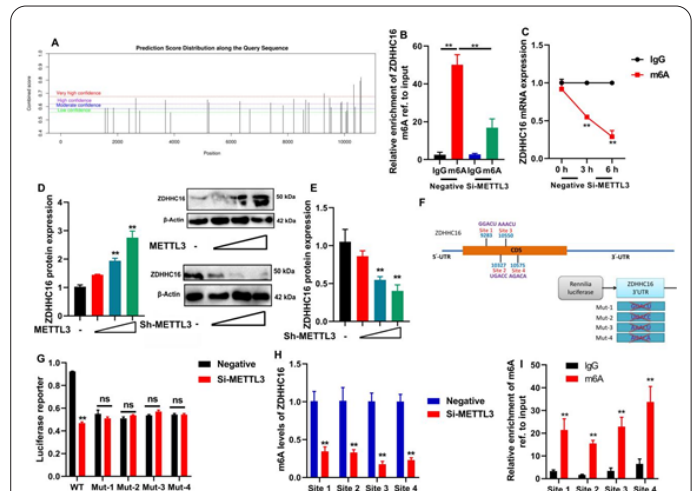


Fig. 9. METTL3-mediated m6A modification increases ZDHHC16 stability. m6A prediction website SRAMP (A), ZDHHC16 mRNA enrichment by METTL3 knockout (B), the stability of ZDHHC16 mRNA (C), METTL3 protein expression (D and E), m6A sites in the 3'-untranslated region (UTR) of METTL3/m6A enrichment of METTL3 levels (F), luciferase activity level (G), m6A enrichment of ZDHHC16 (H and I). Vector, negative control group; ZDHHC16, over-expression of ZDHHC16 group; Si-nc, si-negative control group; Si-ZDHHC16, down-regulation of ZDHHC16 group; **P<0.01 compared with negative control group or si-negative control group.

patients is approximately 4-17%, depending on the stage and region [24]. Considering the different subtypes of lung cancer, targeted therapy is also increasingly used in the personalized treatment of patients [25]. Based on a large number of studies, established risk factors for NSCLC may affect the expression levels of lncRNA in NSCLC tissues and cell lines, thereby affecting the occurrence, development and other malignant phenotypes of NSCLC [25]. In this study, there was an increase of ZDHHC16 mRNA expression in patients with NSCLC. DFS and OS of ZDHHC16 lower expression in patients with NSCLC was higher than those of ZDHHC16 higher expression. ZDHHC16 gene promoted cell Proliferation and Metasta-

sis, and increased the Warburg effect of NSCLC. Xu et al. showed that ZDHHC16 events are associated with survival of Hepatocellular carcinoma patients [26]. Therefore, ZDHHC16 was one oncogenic gene for NSCLC progression through the Warburg effect.

Ferroptosis is a new type of programmed cell death that is oxidative, iron-dependent, and distinct from apoptosis, necrosis, and autophagy [27]. Numerous studies have shown that ferroptosis is associated with various human diseases, including cancer, neurodegenerative diseases, ischemic reperfusion injury, and renal degeneration [28, 29]. The glutathione peroxidase 4 (GPX4) pathway plays a central role in regulating ferroptosis, as evidenced by the cysteine/glutamate reverse transporter system X [30, 31]. In this study, the ZDHHC16 gene reduced ferroptosis of NSCLC by the rehabilitation of mitochondrial structure. Cao et al. revealed that ZDHHC16 played a key role in the early stages of DNA damage responses [16]. Taken together, our data suggest that ZDHHC16 reduced ferroptosis of NSCLC by the rehabilitation of mitochondrial structure.

CREB, as a key signaling pathway for cell regeneration, can promote cell growth [14]. In addition, CREB can also form a positive feedback circuit with BDNF/TrkB signal [13]. TrkB signal can activate CREB through phosphorylation, and the phosphorylated CREB will then activate BDNF to enhance the TrkB signal, thereby enhancing the antioxidant capacity of the body and reducing the rate of neuronal apoptosis [12]. CAMP response element binding protein (CREB), as a transcription factor, has been widely studied in the field of long-term memory formation and the occurrence and treatment of various malignant tumors [32]. We demonstrated that ZDHHC16 promoted CREB expression and interaction between ZDHHC16 protein and CREB protein. Li et al. revealed that ZDHHC16 reduced p-CREB expression in bone marrow mesenchymal stem [18]. Wang et al. revealed that CREB inhibits ferroptosis in lung adenocarcinoma by the inhibition of GPX4 [33]. Therefore, ZDHHC16 promoted CREB expression to reduce ferroptosis of NSCLC.

Ubiquitination modification, as one of the major post-translational modifications of proteins in a eukaryotic cell, mediates the specific degradation of proteins in cells through the ubiquitin-proteasome system (UPS), and extensively participates in and regulates almost all life activity processes such as gene transcription, signal transduction, DNA damage and repair, cell cycle regulation, stress response and even individual immune response in cells [34-36]. The precise regulation of the ubiquitin-proteasome system constitutes a stable and complex ubiquitination signal network, and its imbalance often leads to the occurrence and development of various diseases such as cancer, neurodegenerative diseases, metabolic diseases, etc. [37]. Our study establishes that ZDHHC16 up-regulation reduced CREB Ubiquitination, and down-regulation of ZDHHC16 promoted CREB Ubiquitination of NSCLC. Furthermore, ZDHHC16 reduced CREB Ubiquitination to induce CREB expression of NSCLC, which may be critical for the suppression of ferroptosis of NSCLC.

Previous studies on epigenetics and non-small cell lung cancer have found a close relationship between the two [38, 39]. Epigenetic modifications often interfere with the expression and function of normal genes through DNA/RNA methylation, histone modifications, and changes in the three-dimensional conformation of chromosomes,

thereby affecting the occurrence, development, and drug resistance of tumors [40]. Among them, N6 methyladenosine (m6A) is the most common type of RNA modification and plays an important role in tissue development, stem cell self-renewal and differentiation, and tumor resistance [41]. Therefore, early screening of lung cancer can effectively improve the survival rate of lung cancer patients and research related to m6A is of great significance [42]. It not only relates to the early diagnosis of lung cancer, but may even initiate minimally invasive diagnosis, and also relates to the research of new targeted drugs, making contributions to lung cancer medication [43]. The methylation process of m6A is mainly regulated by three proteins: methyltransferase (writer), demethylase (eraser), and methyl binding protein (also known as recognition protein, reader) [44]. M6Awriter is a type of enzyme that can promote the formation of m6A methylation, among which the earliest discovered m6Awriter methyltransferase 3 (METTL3) has the effect of promoting m6A formation, but cannot methylate rRNA within mRNA; Methyl transferase like 4 (METTL4) and METTL3 are homologous proteins that also promote the formation of m6A modifications [45]. In addition, as an effective component of the m6A writer complex, METTL14 can react with METTL3 to hybridize, and it is also an RNA linker protein that can enhance the activity of m6Awriter [46]. We found that METTL3-mediated m6A modification increases ZDHHC16 stability.

In conclusion, we have found that ZDHHC16 was one oncogenic gene for NSCLC progression. Importantly, m6A methylation/ ZDHHC16 advanced cell proliferation and metastasis of NSCLC by the inhibition of CREB Ubiquitination. ZDHHC16 may provide new clues for understanding the molecular mechanism of NSCLC progress.

Disclosure of conflict of interest

There are no conflicts of interest to declare.

Institutional review board statement

The study was approved by the Ethics Committee of Affiliated Hospital of Shandong University of Traditional Chinese Medicine.

Informed consent statement

Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request

Author contributions

Z.Y.L. and C.Q.J. analyzed data, and wrote the paper; Z.Y.L. and C.Q.J. performed research; Z.Y.L. and W.Z. arranged for obtaining patient informed consents and data collection; Z.Y.L. and W.Z. designed and reviewed the work. The manuscript was critically revised by all authors.

Funding statement

This work was supported by the National Natural Science Foundation of China (grant number: 81874442) and Shandong Provincial Natural Science Foundation (grant number: ZR2021LZY031).

References

- Bhavya K, Mantipally M, Roy S, Arora L, Badavath VN, Gangireddy M et al (2022) Novel imidazo[1,2-a]pyridine derivatives induce apoptosis and cell cycle arrest in non-small cell lung cancer by activating NADPH oxidase mediated oxidative stress. *Life Sci* 294:120334. doi: 10.1016/j.lfs.2022.120334
- Gou W, Yu X, Wu S, Wu H, Chang H, Chen L et al (2022) Targeted inhibition of acidic nucleoplasmic DNA-binding protein 1 enhances radiosensitivity of non-small cell lung cancer. *Cancer Lett* 530:100-109. doi: 10.1016/j.canlet.2022.01.020
- Li M, Liu P, Wang B, Zhou J, Yang J (2022) Inhibition of Nuclear Factor Kappa B as a Therapeutic Target for Lung Cancer. *Altern Ther Health Med* 28:44-51.
- Liu XY, Wei DG, Li RS (2022) Capsaicin induces ferroptosis of NSCLC by regulating SLC7A11/GPX4 signaling in vitro. *Sci Rep* 12:11996. doi: 10.1038/s41598-022-16372-3
- Lu X, Kang N, Ling X, Pan M, Du W, Gao S (2021) MiR-27a-3p Promotes Non-Small Cell Lung Cancer Through SLC7A11-Mediated-Ferroptosis. *Front Oncol* 11:759346. doi: 10.3389/fonc.2021.759346
- Tang X, Ding H, Liang M, Chen X, Yan Y, Wan N et al (2021) Curcumin induces ferroptosis in non-small-cell lung cancer via activating autophagy. *Thorac Cancer* 12:1219-1230. doi: 10.1111/1759-7714.13904
- Kim JW, Min DW, Kim D, Kim J, Kim MJ, Lim H et al (2023) GPX4 overexpressed non-small cell lung cancer cells are sensitive to RSL3-induced ferroptosis. *Sci Rep* 13:8872. doi: 10.1038/s41598-023-35978-9
- Wohlhieter CA, Richards AL, Uddin F, Hulton CH, Quintanal-Villalonga A, Martin A et al (2020) Concurrent Mutations in STK11 and KEAP1 Promote Ferroptosis Protection and SCD1 Dependence in Lung Cancer. *Cell Rep* 33:108444. doi: 10.1016/j.celrep.2020.108444
- Liu M, Fan Y, Li D, Han B, Meng Y, Chen F et al (2021) Ferroptosis inducer erastin sensitizes NSCLC cells to celastrol through activation of the ROS-mitochondrial fission-mitophagy axis. *Mol Oncol* 15:2084-2105. doi: 10.1002/1878-0261.12936
- Feng Y, Xu J, Shi M, Liu R, Zhao L, Chen X et al (2022) COX7A1 enhances the sensitivity of human NSCLC cells to cystine deprivation-induced ferroptosis via regulating mitochondrial metabolism. *Cell Death Dis* 13:988. doi: 10.1038/s41419-022-05430-3
- Shi G, Ni Q, Miao Y, Huang H, Yin Z, Shi W et al (2023) Identification of WD-Repeat Protein 72 as a Novel Prognostic Biomarker in Non-Small-Cell Lung Cancer. *Mediators Inflamm* 2023:2763168. doi: 10.1155/2023/2763168
- Chen Z, Li JL, Lin S, Cao C, Gimbrone NT, Yang R et al (2016) cAMP/CREB-regulated LINC00473 marks LKB1-inactivated lung cancer and mediates tumor growth. *J Clin Invest* 126:2267-2279. doi: 10.1172/JCI85250
- Kang J, Chun J, Hwang JS, Pan C, Li J, Boese AC et al (2022) EGFR-phosphorylated GDH1 harmonizes with RSK2 to drive CREB activation and tumor metastasis in EGFR-activated lung cancer. *Cell Rep* 41:111827. doi: 10.1016/j.celrep.2022.111827
- Tang D, Zhao YC, Qian D, Liu H, Luo S, Patz EF et al (2020) Novel genetic variants in HDAC2 and PPARGC1A of the CREB-binding protein pathway predict survival of non-small-cell lung cancer. *Mol Carcinog* 59:104-115. doi: 10.1002/mc.23132
- Abrami L, Dallavilla T, Sandoz PA, Demir M, Kunz B, Savoglidis G et al (2017) Identification and dynamics of the human ZDHHC16-ZDHC6 palmitoylation cascade. *Elife* 6:e27826. doi: 10.7554/eLife.27826
- Cao N, Li JK, Rao YQ, Liu H, Wu J, Li B et al (2016) A potential role for protein palmitoylation and zDHHC16 in DNA damage response. *Bmc Mol Biol* 17:12. doi: 10.1186/s12867-016-0065-9
- Fan X, Sun S, Yang H, Ma H, Zhao C, Niu W et al (2022) SETD2 Palmitoylation Mediated by ZDHHC16 in Epidermal Growth Factor Receptor-Mutated Glioblastoma Promotes Ionizing Radiation-Induced DNA Damage. *Int J Radiat Oncol Biol Phys* 113:648-660. doi: 10.1016/j.ijrobp.2022.02.018
- Li Z, Cheng Y, Jin X, Wang F, Wang X, Liu S et al (2023) ZDHHC16 restrains osteogenic differentiation of bone marrow mesenchymal stem cells by inhibiting phosphorylation of CREB. *Heliyon* 9:e12788. doi: 10.1016/j.heliyon.2022.e12788
- Chen CJ, Huang JY, Huang JQ, Deng JY, Shanguan XH, Chen AZ et al (2023) Metformin attenuates multiple myeloma cell proliferation and encourages apoptosis by suppressing METTL3-mediated m6A methylation of THRAP3, RBM25, and USP4. *Cell Cycle* 22:986-1004. doi: 10.1080/15384101.2023.2170521
- Fu Y, Liu L, Wu H, Zheng Y, Zhan H, Li L (2023) LncRNA GAS5 regulated by FTO-mediated m6A demethylation promotes autophagic cell death in NSCLC by targeting UPF1/BRD4 axis. *Mol Cell Biochem* doi: 10.1007/s11010-023-04748-6
- Gong S, Wang S, Shao M (2022) Mechanism of METTL14-mediated m(6)A modification in non-small cell lung cancer cell resistance to cisplatin. *J Mol Med (Berl)* 100:1771-1785. doi: 10.1007/s00109-022-02268-2
- Cao Y, Di X, Cong S, Tian C, Wang Y, Jin X et al (2023) RBM10 recruits METTL3 to induce N6-methyladenosine-MALAT1-dependent modification, inhibiting the invasion and migration of NSCLC. *Life Sci* 315:121359. doi: 10.1016/j.lfs.2022.121359
- Cheng X (2022) Effects of Lung Rehabilitation Therapy in Improving Respiratory Motor Ability and Alleviating Dyspnea in Patients with Lung Cancer After Lobectomy: A Clinical Study. *Altern Ther Health Med* 28:18-23.
- Pietrzak J, Szmajda-Krygier D, Wosiak A, Swiechowski R, Michalska K, Mirowski M et al (2022) Changes in the expression of membrane type-matrix metalloproteinases genes (MMP14, MMP15, MMP16, MMP24) during treatment and their potential impact on the survival of patients with non-small cell lung cancer (NSCLC). *Biomed Pharmacother* 146:112559. doi: 10.1016/j.biopha.2021.112559
- Ran H, Ma J, Cai L, Zhou H, Yuan Z, Chen Y et al (2022) Serum cholinesterase may independently predict prognosis in non-small-cell lung cancer. *Bmc Cancer* 22:93. doi: 10.1186/s12885-022-09212-0
- Xu Q, Xu H, Deng R, Li N, Mu R, Qi Z et al (2021) Immunological significance of prognostic alternative splicing signature in hepatocellular carcinoma. *Cancer Cell Int* 21:190. doi: 10.1186/s12935-021-01894-z
- Pu Z, Sui B, Wang X, Wang W, Li L, Xie H (2023) The effects and mechanisms of the anti-COVID-19 traditional Chinese medicine, Dehydroandrographolide from *Andrographis paniculata* (Burm.f.) Wall, on acute lung injury by the inhibition of NLRP3-mediated pyroptosis. *Phytomedicine* 114:154753. doi: 10.1016/j.phymed.2023.154753
- Zou J, Wang L, Tang H, Liu X, Peng F, Peng C (2021) Ferroptosis in Non-Small Cell Lung Cancer: Progression and Therapeutic Potential on It. *Int J Mol Sci* 22:13335. doi: 10.3390/ijms222413335
- Cheng F, Dou J, Yang Y, Sun S, Chen R, Zhang Z et al (2023) Drug-induced lactate confers ferroptosis resistance via p38-SGK1-NEDD4L-dependent upregulation of GPX4 in NSCLC cells. *Cell Death Discov* 9:165. doi: 10.1038/s41420-023-01463-5
- Zhang N, Wu Y, Wu Y, Wang L, Chen J, Wang X et al (2022) Ferroptosis-Related Genes Are Potential Therapeutic Targets and the Model of These Genes Influences Overall Survival of NSCLC Patients. *Cells* 11:2207. doi: 10.3390/cells11142207
- Cui X, Liu C, Dong P, Liu C, Bai Y (2023) The combination

- therapy of isomucronulatol 7-O-beta-glucoside (IMG) and CEP-9722 targeting ferroptosis-related biomarkers in non-small cell lung cancer (NSCLC). *Bmc Pulm Med* 23:162. doi: 10.1186/s12890-023-02445-0
32. Xia Y, Zhan C, Feng M, Leblanc M, Ke E, Yeddula N et al (2018) Targeting CREB Pathway Suppresses Small Cell Lung Cancer. *Mol Cancer Res* 16:825-832. doi: 10.1158/1541-7786.MCR-17-0576
33. Wang Z, Zhang X, Tian X, Yang Y, Ma L, Wang J et al (2021) CREB stimulates GPX4 transcription to inhibit ferroptosis in lung adenocarcinoma. *Oncol Rep* 45:88. doi: 10.3892/or.2021.8039
34. Han T, Guo M, Gan M, Yu B, Tian X, Wang JB (2018) TRIM59 regulates autophagy through modulating both the transcription and the ubiquitination of BECN1. *Autophagy* 14:2035-2048. doi: 10.1080/15548627.2018.1491493
35. Li K, Zheng X, Tang H, Zang YS, Zeng C, Liu X et al (2021) E3 ligase MKRN3 is a tumor suppressor regulating PABPC1 ubiquitination in non-small cell lung cancer. *J Exp Med* 218:e20210151. doi: 10.1084/jem.20210151
36. Wang G, Long J, Gao Y, Zhang W, Han F, Xu C et al (2019) SETDB1-mediated methylation of Akt promotes its K63-linked ubiquitination and activation leading to tumorigenesis. *Nat Cell Biol* 21:214-225. doi: 10.1038/s41556-018-0266-1
37. De S, Holvey-Bates EG, Mahen K, Willard B, Stark GR (2021) The ubiquitin E3 ligase FBXO22 degrades PD-L1 and sensitizes cancer cells to DNA damage. *Proc Natl Acad Sci U S A* 118:e2112674118. doi: 10.1073/pnas.2112674118
38. Ma Y, Yang J, Ji T, Wen F (2022) Identification of a novel m5C/m6A-related gene signature for predicting prognosis and immunotherapy efficacy in lung adenocarcinoma. *Front Genet* 13:990623. doi: 10.3389/fgene.2022.990623
39. Pei H, Dai Y, Yu Y, Tang J, Cao Z, Zhang Y et al (2023) The Tumorigenic Effect of lncRNA AFAP1-AS1 is Mediated by Translated Peptide ATMLP Under the Control of m(6) A Methylation. *Adv Sci (Weinh)* 10:e2300314. doi: 10.1002/advs.202300314
40. Ren S, Xiao Y, Yang L, Hu Y (2023) RNA m6A methyltransferase METTL14 promotes the procession of non-small cell lung cancer by targeted CSF1R. *Thorac Cancer* 14:254-266. doi: 10.1111/1759-7714.14741
41. Wang X, Hu Y, Li X, Zhu C, Chen F (2023) YTHDC2-mediated m6A mRNA modification of Id3 suppresses cisplatin resistance in non-small cell lung cancer. *J Thorac Dis* 15:1247-1257. doi: 10.21037/jtd-23-187
42. Wu L, Cheng D, Yang X, Zhao W, Fang C, Chen R et al (2022) M2-TAMs promote immunoresistance in lung adenocarcinoma by enhancing METTL3-mediated m6A methylation. *Ann Transl Med* 10:1380. doi: 10.21037/atm-22-6104
43. Lin X, Ye R, Li Z, Zhang B, Huang Y, Du J et al (2023) KIAA1429 promotes tumorigenesis and gefitinib resistance in lung adenocarcinoma by activating the JNK/ MAPK pathway in an m(6)A-dependent manner. *Drug Resist Updat* 66:100908. doi: 10.1016/j.drug.2022.100908
44. Zhao S, Song P, Zhou G, Zhang D, Hu Y (2023) METTL3 promotes the malignancy of non-small cell lung cancer by N6-methyladenosine modifying SFRP2. *Cancer Gene Ther* 30:1094-1104. doi: 10.1038/s41417-023-00614-1
45. Zhou Q, Lai X, Gao Y, Chen Q, Xu Y, Liu Y (2023) METTL14 Regulates PLAGL2/beta-Catenin Signaling Axis to Promote the Development of Nonsmall Cell Lung Cancer. *J Oncol* 2023:4738586. doi: 10.1155/2023/4738586
46. Yuan B, Qin H, Zhang J, Zhang M, Yang Y, Teng X et al (2022) m(6)A regulators featured by tumor immune microenvironment landscapes and correlated with immunotherapy in non-small cell lung cancer (NSCLC). *Front Oncol* 12:1087753. doi: 10.3389/fonc.2022.1087753